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PROTEINASE PRODUCTION IN RELATION TO GROWTH OF A MICROCOCCUS SPECIES[1]

I. J. McDONALD

Abstract

Production of proteinase(s) by a *Micrococcus* sp. (A.T.C.C. No. 407) in general was related to the amount of growth. However, addition of 2% sodium chloride to tryptone yeast extract broth resulted in an apparent stimulation of proteinase production without an increase in growth. The salt apparently protected the enzyme since it was found that proteinase preparations were inactivated less rapidly in the presence than in the absence of salt. Although the organism did not require carbohydrate for growth, it utilized maltose but not glucose or other carbohydrates. In the presence of maltose, growth and proteinase production were stimulated. The organism produced proteinase on a minimal synthetic medium containing glutamic acid as the sole source of carbon and nitrogen.

Introduction

In a previous study, proteinase was demonstrated in cell-free supernatants of cultures of *Micrococcus freudenreichii* and some characteristics of the partially purified enzyme were described (12). Later investigations cast doubt on the identification of the organism and it is referred to in this and other papers (14) as *Micrococcus* sp. Attempts to obtain proteinase from non-proliferating cells of *Micrococcus* sp. by incubating heavy suspensions in liquid media for several hours, as has been done with *Streptococcus liquefaciens* (17), were unsuccessful. Consequently a study was made of the effects of time and temperature of incubation and of composition of the medium to determine if enzyme production could be affected independently of growth. The results of this study are presented here.

Materials and Methods

Cultures of *Micrococcus* sp. (*M. freudenreichii* A.T.C.C. No. 407) were maintained by monthly transfer on blood agar base (Difco) slopes. Unless

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otherwise stated, factors affecting enzyme production were determined using a basal medium of tryptone yeast extract broth (tryptone (Difco) 0.1%, yeast extract (Difco) 0.1%) at pH 6.5. This medium containing 1.9% tris(hydroxymethyl) aminomethane (tris) was used in experiments on the effect of the pH of growth medium. Anaerobic conditions were obtained by evacuating the atmosphere above flasks, replacing it with the required gas, and repeating the procedure four times. The synthetic media employed have been described previously (14).

Tryptone yeast extract media were inoculated with 1 or 2% (v/v) of a 20- to 24-hour culture of the organism in tryptone yeast extract broth; synthetic media were inoculated with washed cells (14). All media were incubated at 25° C unless otherwise noted on a rotating platform shaker operating at approximately 100 r.p.m.

Growth was expressed as optical density of cultures at 660 mμ (14). Anthrone was used to determine the carbohydrate content of media (15). Proteinase activity was measured at 37° C by incubating 5.0 ml of supernatant, obtained by centrifugation of cultures (10,000 $\times$ *g* at 0° C) with 5.0 ml of casein substrate and 0.1 ml of toluene. The casein substrate (12) was modified by adding 30 ml of 0.05 *N* monopotassium phosphate after boiling and then by adjusting to pH 5.7 with hydrochloric acid. Where necessary, tubes were adjusted to contain the same amount of salt. Samples of these reaction mixtures, taken at 0 and 4 hours, were mixed thoroughly with an equal volume of 0.6 *N* trichloroacetic acid (TCA) and filtered through Whatman No. 2 filter paper. Tyrosine was determined in 1.0 ml of the TCA filtrate by the method of Anson (1). One unit of enzyme was defined as the amount of enzyme which liberated 1.0 μg of tyrosine under these conditions.

The effect of sodium chloride on enzyme inactivation was determined using supernatants obtained from 40-hour cultures in tryptone yeast extract broth and preserved by the addition of 0.2 mg of merthiolate per 100 ml. Enzyme activity was measured in samples held at 4° C or incubated at 25° C on a shaker.

Results

Proteolytic enzyme(s) produced by *Micrococcus* sp. in tryptone yeast extract broth was demonstrated in cell-free supernatants obtained by centrifugation but not in Seitz-filtrates. The amount of proteinase produced was related to the amount of growth (Table I). When the atmosphere in the flask was carbon-dioxide-free air (i.e. air passed over ascarite) growth of the organism was less than in air; when the atmosphere was nitrogen only limited growth occurred and, in other experiments, when care was taken to insure absolutely anaerobic conditions, no growth occurred. The organism, therefore, requires oxygen for growth and small amounts of carbon dioxide appear to be stimulatory. (The differences in amount of growth in air between experiments A and B can be accounted for by differences in media, inocula, rates of aeration, etc.)

The initial pH of the medium affected growth of the organism. In tryptone yeast extract broth containing tris buffer the optimal range for growth was pH 5.5–6.5. Subsequent experiments were performed using tryptone yeast extract broth adjusted to pH 6.0–6.5.

TABLE I

Effect of different atmospheres on growth of and proteinase production by *Micrococcus* sp.*
(40 hours at 25° C)

Atmosphere	Growth (O.D. at 660 mμ)	Proteinase (units)
Experiment A		
Air	0.67	31
N_2	0.12	5
Experiment B		
Air	0.46	17
CO_2-free air	0.36	12

*Medium = tryptone yeast extract broth. Data representative of three trials.

Growth of the organism was measured at various temperatures. At 25° C the organism grew rapidly and produced maximal amounts of enzyme in the shortest time (Table II). On extended incubation at 10 and 15° C the organism produced moderately large amounts of enzyme whereas prolonged incubation at 25° C and at 32° C caused a decrease in enzyme activity. The results suggest that the optimum temperature for growth and enzyme production was 25° C.

Growth of *Micrococcus* sp. was stimulated slightly at low concentrations of sodium chloride (Table III). Proteinase production, however, appeared to be optimal when the medium contained 2% sodium chloride. Other salts of sodium and potassium (i.e. $NaNO_3$.Na_2SO_4, KCl, KNO_3, KBr, and KI), when added to the medium at a concentration of about 0.25 *M*, gave a similar apparent stimulation of proteinase production. Cell-free preparations of proteinase were incubated in the absence and in the presence of sodium chloride. The results (Table IV) indicate that salt reduced the rate of inactivation of the enzyme but did not prevent it completely. Even at refrigeration temperature, considerable inactivation of the enzyme occurred in the absence of salt.

Although *Micrococcus* sp. grew in a medium without added carbohydrates, the possibility that such substances might influence growth and proteinase production was investigated. In tryptone yeast extract broth, arabinose, ribose, xylose, rhamnose, fructose, galactose, glucose, mannose, cellobiose, lactose, melibiose, sucrose, trehalose, raffinose, α-methyl glucose, mannitol, or salicin were not utilized and none had significant effect on growth. Maltose, however, was utilized by the organism and growth with maltose was significantly greater than it was with glucose (Table V).

The addition of maltose to a minimal synthetic medium resulted in its utilization and in marked stimulation of growth and proteinase production (Table VI). The organism, however, was capable of slight growth and some proteinase production in the minimal medium with glutamic acid as the sole source of carbon and nitrogen.

Components of a synthetic amino acid medium (14) were investigated for their effects on proteinase production. Single amino acids and vitamins could

TABLE II

Effect of temperature of incubation on growth of and proteinase production by *Micrococcus* sp.*

Incubation time (hr)	Temperature							
	10° C		15° C		25° C		32° C	
	Growth† (O.D.)	Proteinase (units)	Growth† (O.D.)	Proteinase (units)	Growth† (O.D.)	Proteinase (units)	Growth† (O.D.)	Proteinase (units)
17	0.13	0.0	0.35	4.0	0.77	29.0	0.79	23.5
41	0.44	7.0	0.52	7.0	0.94	44.5	0.84	24.5
65	0.73	21.0	0.94	34.5	0.89	39.0	0.96	19.0
89	0.88	31.0	0.92	35.5	0.87	35.0	0.76	16.0

*Medium = tryptone yeast extract broth. Data representative of three trials.
†O.D. measured at 660 mμ.

be omitted from the medium without any significant effect on proteinase production except for those that influenced growth i.e. glutamic acid, glycine, methionine, or thiamine (14). Omission of these resulted in a reduction in proteinase production concomitant with a reduction in growth.

TABLE III

Effect of sodium chloride on growth of and proteinase production by *Micrococcus* sp.*
(40 hours at 25° C)

Added sodium chloride (g/100 ml)	Growth (O.D. at 660 mμ)	Proteinase (units)
0.0	0.72	16
0.25	0.76	56
0.5	0.76	63
1.0	0.70	83
2.0	0.65	96
4.0	0.32	35
6.0	0.12	6

*Grown in tryptone yeast extract broth plus sodium chloride as shown. Data representative of three trials.

TABLE IV

Effect of sodium chloride on inactivation of *Micrococcus* sp. proteinase*

Incubation temp.	Time (hr)	Enzyme activity			
		0% NaCl		4% NaCl	
		Units	% loss	Units	% loss
4° C (stationary)	20	78	0.0	80	0.0
	42	77	2.0	78	2.5
	66	62	20.0	75	6.0
37° C (shaken)	20	6	92.4	59	27.0
	42	3	96.2	39	51.0
	66	1	98.8	20	75.0

*Enzyme obtained by centrifuging a 40-hour culture in tryptone yeast extract broth and preserved by adding 2.0 mg of merthiolate per 100 ml of supernatant. Data representative of three trials.

TABLE V

Utilization of maltose and glucose and their effects on growth of *Micrococcus* sp.* (25° C)

Incubation time (hr)	Maltose		Glucose	
	Conc. (mg/ml)	Growth (O.D. at 660 mμ)	Conc. (mg/ml)	Growth (O.D. at 660 mμ)
21	5.50	0.18	5.32	0.06
40	4.56	0.66	5.34	0.34
64	3.75	0.80	5.05	0.37

*Carbohydrates sterilized separately and 0.5% added to tryptone yeast extract broth. Data representative of several trials.

TABLE VI

Utilization of maltose and its effect on growth of and proteinase production by *Micrococcus* sp.*
(40 hours at 25° C)

Maltose concentration (mg/ml)		Growth (O.D. at 660 mμ)	Proteinase (units)
Initial	Final		
0.0	0.0	0.12	14
0.5	0.03	0.48	61
1.0	0.49	0.51	67
2.0	1.16	0.60	86
5.0	3.46	0.82	109

*Basal medium = $MgSO_4$ 25 mg, KH_2PO_4 100 mg, NaCl 0.9 g, thiamine 200 μg, biotin 4.0 μg, glutamic acid 0.5 g per 100 ml. Maltose sterilized separately. Data representative of two trials.

Discussion

Many bacteria have been shown to elaborate proteinases in direct relation to the amount of growth (5, 7, 9, 16, 18) and *Micrococcus* sp. can be added to this group. At least one bacterial species, *Streptococcus liquefaciens*, has been shown to produce proteinase in the absence of cell multiplication (17) and enzyme production depended upon the presence of certain amino acids, vitamins, carbohydrates, purines, and pyrimidines (11, 17). Attempts to show similar effects using similar agents with *Micrococcus* sp. were unsuccessful. Proteinase production by this organism was altered only with a corresponding alteration of the amount of growth. Although adding 2% sodium chloride to the medium resulted in an apparent stimulation of enzyme production without an increase in the amount of growth, it was shown that cell-free enzyme preparations were not inactivated as rapidly in the presence as in the absence of sodium chloride, suggesting that the proteinase required a solution of appropriate ionic strength to maintain activity. There does not seem to be any evidence for specific ion requirements to prevent loss of activity as shown for the proteinase of *Micrococcus lysodeikticus* (8) or for the isocitric dehydrogenase of a marine bacterium (13). The proteinase behaved somewhat like the lactic dehydrogenase of *Halobacterium salinarium*, which was protected against inactivation by a variety of salts (2), although at much higher salt concentrations than those used here.

Physiologically *Micrococcus* sp. is a rather interesting organism. Although it is capable of growth without carbohydrate, it utilizes maltose but not glucose or other mono- or di-saccharides. These results indicate that the organism may possess a permease (4) specific for maltose and suggest that its carbohydrate metabolism should be examined in greater detail. Also, the organism grows and produces proteinase in a medium containing glutamic acid as the sole carbon and nitrogen source indicating that protein is not essential for proteinase production. Dependence of proteinase elaboration on the presence of protein has been shown for some bacteria (3,6) but not for others (10, 17).

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USE OF BETA-IONONE IN ENRICHMENT CULTURES FOR ISOLATING PARACOLOBACTRUM AND CERTAIN FUNGI FROM SOIL[1]

JAMES E. ZAJIC AND HAROLD H. KUEHN

Abstract

Beta-ionone, an acyclic terpene used extensively in perfumery, was used in enrichment media for isolation and study of certain soil microorganisms. Two-tenths per cent beta-ionone inhibited the growth of about 99% of the bacterial and fungal population. Fungi were more sensitive than bacteria to beta-ionone. The most frequent bacterial isolates obtained from media enriched with beta-ionone were representatives of the genus *Paracolobactrum*. A few of these isolates were identical with the type species, *P. aerogenoides*, but the majority differed by their ability to liquefy gelatin. This difference may provide an adequate basis for establishing a new species in this genus which, because of its similarity to *Aerobacter cloacae*, tentatively may be designated as *Paracolobactrum cloacanoides nomen provisorium*. Other coliform bacteria were isolated but with reduced frequency. *Fusarium* species and *Trichoderma viride* were the fungi isolated most frequently from soil samples plated on beta-ionone-enriched media. *Geotrichum candidum* and *Scopulariopsis brevicaulis* were isolated from specific soils using this enrichment procedure. A single isolate from soil was made of *Curvularia pallescens*, which previously has been isolated only once, from soil in India.

Introduction

There are only a few reports in the literature concerning the influence of essential oils and their constituent terpenes on microbes. In 1958 Maruzzella and Henry (4) examined the antimicrobial properties of 100 essential oils with regard to their effect on 10 bacteria and fungi. They found essential oils to possess remarkable antimicrobial properties against both bacteria and fungi. Recently Ark and Thompson (1) observed that an organic solvent extract from garlic could be used to control nine different microbial plant diseases. Such indications of antimicrobial characteristics of terpenes were instrumental in stimulating the following investigation. Beta-ionone, an acyclic monoterpenoid, was the principal terpene studied; it was added to enrichment media and its influence as a selective agent for certain microbes from soils studied.

Materials and Methods

Cultivated and non-cultivated soils were collected at random in the following localities: Muscatine, Iowa; Washington, D.C.; Miami Beach, Florida; Titusville, Florida; Killearn Gardens, Florida; Montgomery, Alabama; and Corinth, Mississippi. No endeavor was made to identify the specific types of soils. Two different beta-ionone-enriched media were used. Medium 1 contained 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.05% KNO_3, 0.01% yeast extract, 0.04% peptone, 3.0% glucose, and beta-ionone in concentrations of

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Contribution from the Research Department, Grain Processing Corporation, Muscatine, Iowa.

0.2–1.6% at pH 7.0. Medium 2 was identical with medium 1 except that an hydrolyzate of soybean protein, phytone, was substituted for peptone. Microbial counts of the soils were made using standard plating methods. Media and techniques used in identifying bacteria were those described in the official Manual of Microbiological Methods (5) accepted by the Society of American Bacteriologists. The fungi were subcultured on a malt-extract agar for identification.

Experiments were conducted at pH levels of 5.0 and 7.0 in order to obtain a slightly broader spectrum of the influence of beta-ionone on growth of bacteria and fungi found in soil. Usually, 1:100 to 1:10,000 dilutions of soil samples were adequate for plate counts. Bacterial and fungal counts were made after 4 days' incubation of the enrichment plates at 30° C. Growth of the fungi was slow, and it was advantageous to recount the fungal colonies after 6 days.

Results

Table I presents a compilation of the data with one soil from Muscatine, Iowa, in a typical experiment. Medium 1, with or without beta-ionone, appeared to be superior for growth of bacteria, while the fungi grew equally well on both media. Results illustrate that beta-ionone had pronounced antimicrobial activity against both bacteria and fungi. In both medium 1 and 2 at pH 5.0 and 7.0, 0.2% beta-ionone inhibited the growth of at least 99.8% of the bacteria. Under the same conditions this concentration of beta-ionone inhibited growth of more than 99.2% of the fungi found in this soil sample. Except in medium 1 at pH 7.0 complete inhibition of fungal growth was observed with 0.8% or more of beta-ionone. Bacteria were more tolerant to beta-ionone than were fungi. With but one exception, at least some bacterial growth was observed with 1.6% beta-ionone, which was the highest level tested, whereas at this concentration fungi rarely developed.

TABLE I

The influence of beta-ionone on the bacteria and fungal populations of soil

	Resistant cells (million per gram of soil)			
	pH 5.0		pH 7.0	
% beta-ionone	Bacteria	Fungi	Bacteria	Fungi
Medium 1 (peptone)				
0.0	210.	1.10	300.	.500
0.2	.100	.0018	.190	.004
0.4	.040	.0014	.100	.002
0.8	.030	—	.087	.0007
1.6	.004	—	.024	.0003
Medium 2 (phytone)				
0.0	120.	7.00	70.0	20.0
0.2	.066	.004	.110	.100
0.4	.042	.0004	.067	—
0.8	.009	—	.064	—
1.6	—	—	.014	—

The frequent occurrence of certain mucoid colony types on the enrichment plates made from every soil examined suggested that beta-ionone might be useful as a selective agent to enable easy isolation of these mucoid bacteria. Such bacteria, characteristically mucoid and varying from transparent to slightly milky in color, outgrew all other bacteria and fungi, becoming microscopically apparent within 24 hours and increasing in size to 0.5–1.5 cm diameter in about 48 hours. Other, smaller bacterial colonies were present but their low frequency of occurrence made them less amenable for study.

Fungal colonies which appeared within 2–3 days exhibited very slow growth on beta-ionone media. Since they did not sporulate on these media, it was necessary to transfer them to other media, lacking beta-ionone, for identification. Since all colonies were transferred and studied the frequency of occurrence of the various genera of fungi was determined (Table II). *Trichoderma viride* and species of *Fusarium* occurred with the highest frequency and were isolated from the largest number of soil samples. Two species of *Aspergillus*, *A. flavus* and *A. ustus*, were isolated with moderate frequency, while *A. niger* and *A. ochraceus* were rarely encountered. *Geotrichum candidum* was very prevalent in a soil sample from Miami Beach, Florida, and this species was also encountered frequently in certain other soils not included in the present study. Species of *Penicillium* were isolated at a moderate frequency. In one soil *Scopulariopsis brevicaulis* was the predominant fungus, but it was completely absent in all other isolations. Other infrequent isolates were *Monilia sitophila*, *Stachybotrys atra*, and *Curvularia pallescens*. The latter species was found in a soil sample from Washington, D.C.; it has been found in soil only once previously in India (6).

A taxonomic study of the predominant mucoid bacteria encountered in the presence of beta-ionone has shown them to be Gram-negative, motile rods possessing characteristics identified with the genus *Paracolobactrum* (2). Pure cultures of *Paracolobactrum* were obtained in a single transfer through careful selection of the largest and most rapidly growing colonies. Sixty-eight colonies, obtained from a soil from Corinth, Mississippi, were selected for identification. No attempt was made to select only the largest, mucoid type of colonies, although, as usual on beta-ionone media, there was a predominance of this group. Using lactose utilization, gelatin liquefaction, IMVIC tests, as well as other conventional taxonomic techniques, 35 of the 68 isolates were identified as falling within the genus *Paracolobactrum* (Table II). Three of these were identical with *P. aerogenoides*, while the remaining 32 were similar to *P. aerogenoides* in the ability to ferment lactose and to produce acetylmethylcarbinol, etc., but were unlike *P. aerogenoides* since they were able to liquefy gelatin. Included in the 68 isolates were 8 isolates of *Aerobacter aerogenes*, 2 of *A. cloacae*, and 1 of *Escherichia freundii*. The 22 remaining isolates were not identified positively; however, they were non-lactose fermenters and formed pellicles on liquid media. Four of these last 22 isolates were similar to *Paracolobactrum arizonae*, 7 resembled *P. coliforme*, and 6 were similar to *P. intermedium*. All differed from *P. aerogenoides* by a negative acetylmethylcarbinol reaction.

The validity of the genus *Paracolobactrum* has been questioned since it differs from the other coliforms in only one biochemical characteristic (3),

i.e. the slow utilization of lactose. To find whether growth in the presence of beta-ionone might provide a second taxonomic characteristic associated with the paracolobacteria, a comparison of growth was made using known cultures of *E. coli* and *A. aerogenes* and an isolate referred to here as "*Paracolobactrum aerogenoides*-like". *A. aerogenes* was inhibited to the greatest extent by beta-ionone (Table III); 0.1% beta-ionone inhibited 63% of the growth. With 0.1,

TABLE II

The frequency of isolation of various bacteria and fungi from soils plated on an enrichment medium containing beta-ionone

		% beta-ionone	
		0.2	0.4
Source of soil	Microorganism	Isolation	Frequency
	I. Bacteria		
Muscatine, Iowa	*Paracolobactrum* (*aerogenoides*-like)	32	NC
	Paracolobactrum aerogenoides	3	NC
	Aerobacter aerogenes	8	NC
	Aerobacter cloacae	2	NC
	Escherichia freundii	1	NC
	Unknown	22	NC
	II. Fungi		
Muscatine, Iowa	*Scopulariopsis brevicaulis*	28	16
	Trichoderma viride	17	19
	Fusarium spp.	5	7
	Aspergillus flavus	1	1
	Monilia sitophila	1	0
	Penicillium sp.	1	0
	Unknown	1	0
Washington, D.C.	*Trichoderma viride*	7	14
	Penicillium spp.	11	7
	Aspergillus flavus	8	6
	Aspergillus ochraceus	1	0
	Fusarium spp.	4	0
	Aspergillus ustus	0	1
	Curvularia pallescens	0	1
	Unknown (yeast)	0	1
Miami Beach, Fla.	*Geotrichum candidum*	10	8
	Fusarium spp.	9	31
	Penicillium spp.	1	4
Titusville, Fla.	*Trichoderma viride*	4	14
	Fusarium spp.	8	9
	Penicillium (three spp.)	7	6
	Stachybotrys atra	1	5
	Aspergillus niger	0	1
Killearn Gardens, Fla.	*Fusarium* spp.	26	NC
Corinth, Miss.	*Penicillium* (two spp.)	18	NC
	Aspergillus ustus	7	NC
	Fusarium spp.	2	NC
	Aspergillus niger	1	NC

NOTE: NC = not counted. Medium 1 was used as the enrichment medium.

TABLE III

Influence of beta-ionone on growth of *Aerobacter aerogenes*, *Escherichia coli*, and an isolate similar to *Paracolobactrum aerogenoides*

% beta-ionone	Dry weight of cells (g/100 ml)		
	Aerobacter aerogenes	*Escherichia coli*	*Paracolobactrum aerogenoides*-like
0.0	.38	.38	.35
0.1	.14	.32	.28
0.5	.07	.31	.28
1.0	.04	.21	.28
2.0	.01	.03	.24

NOTE: Medium 1 was used for this experiment.

0.5, and 1.0% beta-ionone *E. coli* and "*P. aerogenoides*-like" both synthesized about the same amount of tissue. However, at 2.0% beta-ionone growth of *E. coli* was inhibited almost completely whereas the isolate of *Paracolobactrum* was inhibited only about 31%. Resistance to ionone appears to be a characteristic associated with the genus *Paracolobactrum*.

Discussion

Using beta-ionone enrichment media, species of the genus *Paracolobactrum* have been isolated from all soil samples examined. Thus, this procedure would seem to provide an excellent selective mechanism for isolating species of this genus. This contribution may have possible commercial significance since *P. coliforme* has been reported to synthesize valine (7).

Of the 35 isolates of *Paracolobactrum* which were positively identified, only 3 represented *P. aerogenoides*. The remaining 32 isolates liquefied gelatin and thus differed from *P. aerogenoides*. However, since these 32 isolates produced acetylmethylcarbinol they also differed from the other 3 recognized species, *P. arizonae*, *P. intermedium*, and *P. coliforme*. A relationship arises comparable to that between *Aerobacter aerogenes* and *A. cloacae* in which gelatin liquefaction is of primary taxonomic significance. Because of this difference, species standing should be considered for those isolates of *Paracolobactrum* which are identical with *P. aerogenoides* except for the ability to liquefy gelatin. In view of the relationship to *A. cloacae*, this species might be designated as *Paracolobactrum cloacanoides* (*Paracolobactrum cloacanoides nomen provisorium*).

Ewing and Edwards (3) recently stated that they "cannot accept the genus *Paracolobactrum*" because the genus was erected on the basis of a single reaction. The single reaction referred to is the failure to ferment lactose, or to ferment it slowly. The high tolerance of isolates of *Paracolobactrum* to beta-ionone may have value and taxonomic significance in the identification and classification of the coliform bacteria because it affords a second characteristic by which the genus *Paracolobactrum* may be differentiated from other coliforms, such as *Escherichia* and *Aerobacter*. Table III illustrates the results of an experiment which compared the resistance to beta-ionone of *A. aerogenes*, *E. coli*, and the isolates provisionally designated as *Paracolobactrum*

cloacanoides. Although the scope of this particular experiment was limited to single cultures of *A. aerogenes* and *E. coli*, the results reveal an apparent inability of these cultures to withstand a 2% concentration of beta-ionone. If further experimentation verifies these results it would tend to substantiate the retention of *Paracolobactrum* as a generic entity (2) and to refute the contention of Ewing and Edwards (3).

Results have shown fungi to be much more sensitive than bacteria to beta-ionone. In most instances 0.8–1.6% beta-ionone inhibited the growth of all soil fungi. These results would agree with those of Maruzzella and Henry (4), who reported fungi to be almost twice as susceptible as bacteria to perfume oils containing terpenes. These workers also found that *Mucor mucedo* was highly sensitive to perfume oils. Representatives of the Mucorales were completely absent in media enriched with beta-ionone, although these molds were isolated from the tested soils by control platings on malt extract agar. The apparent inability of Mucorales to grow in the presence of beta-ionone suggests a convenient means of excluding these rapidly spreading fungi from plates in routine screening of soil samples.

This study has shown that in addition to having possible taxonomic significance, the use of antimicrobial substances, such as beta-ionone, in enrichment media may serve two purposes. The function as used here has been its inclusion in a technique for the isolation of certain ecological groups of microorganisms resistant to the particular chemical. Second, since most enrichment procedures are highly selective, a determination can be made as to the actual number of cells that a specific genus and (or) species contributes to the total microbial flora of the sample tested. This second purpose has not been pursued to the fullest extent. As these techniques develop they should make a considerable contribution to the complex field of microbial ecology.

Acknowledgment

We wish to thank Dr. A. G. Lochhead, Canada Department of Agriculture, for his initial identification of our isolates of *Paracolobactrum*, and Dr. J. W. Carmichael, Provincial Laboratory of Public Health, University of Alberta, Canada, for verifying the identification of *Geotrichum candidum*.

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BORDETELLA BRONCHISEPTICA BACTERIOPHAGE[1]

HELENE C. RAUCH[2] AND M. J. PICKETT

Abstract

Thirty-eight phages were isolated from 48 strains of *Bordetella bronchiseptica*, and three additional phages were obtained as spontaneous, virulent mutants of these isolates. No evidence of induction was obtained upon exposing lysogenic *B. bronchiseptica* to ultraviolet light. Thirty-five of the 48 strains of *B. bronchiseptica* were sensitive to one or more of the 41 phages, and a unique phage pattern was obtained for each strain. Nine strains of *Bordetella parapertussis* were tested and each was sensitive to at least two of these phages; this heterologous lysis of *B. parapertussis* was obtained with 18 of the *B. bronchiseptica* phages, and represents additional evidence for the taxonomic relationship of these two species. However, no similar relationship was demonstrable with *Bordetella pertussis;* 50 strains were examined but all were insensitive to these phages. Seven strains of *Alcaligenes faecalis* and several of *Brucella* species were equally insensitive.

Introduction

Since its discovery almost 50 years ago, *Bordetella bronchiseptica* has been placed in six different genera (15). Though serological evidence of its relation to *Bordetella pertussis* and *Bordetella parapertussis* has been presented by several workers (2, 8, 10, 11) the present classification of these related species was first firmly established by the nutritional studies of Proom (16). The purpose of this paper is to describe lysogenic strains of *B. bronchiseptica* and to present additional evidence in support of a taxonomic relationship between this microorganism and *B. parapertussis.*

Materials and Methods

Bacterial Cultures

Stock cultures of *Bordetella bronchiseptica* (Table I) and *Alcaligenes faecalis* (strains K-11, K-19, K-20, K-24, K-25, K-26, and K-27) (14) were stored at 4° C on infusion agar slants. The latter were prepared from heart infusion broth (HIB), Difco, solidified with 2% agar. All strains of *B. bronchiseptica* hydrolyzed urea in the medium of Ferguson and Hook (9) within 4 hours at 37° C and none fermented glucose or lactose. Microscopically, all were motile Gram-negative coccobacilli. Colonially, most were smooth to intermediate in type when observed by oblique lighting under a dissecting microscope. However, according to the acriflavine test (5, 6) only strains numbered 40, 42, 43, and 47 were intermediate and all others were rough.

Stock cultures of *B. pertussis** and *B. parapertussis*† were maintained on a

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[2]Present address: Department of Infectious Diseases, School of Medicine, Stanford University, Stanford, California.
*Kindly supplied by Dr. M. White, Cedars of Lebanon Hospital, Los Angeles, California.
†Kindly supplied by Dr. G. Eldering, and by Statens Seruminstitut, Copenhagen, Denmark.

TABLE I

Stock cultures of *Bordetella bronchiseptica*

Stock culture Number	Isolated from:	Source
3	Hog	Grace Eldering, Michigan Dept. of Health, Grand Rapids, Michigan
4	Ferret	
5	Child	
7	Rabbit	
8	Guinea pig	
12	?	
13	?	
20	Dog	J. A. Ulrich, Mayo Clinic, Rochester, Minn.
21	?	
22	Man	
23	Mouse	
24	Guinea pig	
25	?	
26	Dog	
27	Dog	
28	Hog	
29	?	
30	Dog	
31	Hog	
32	Dog	
33	?	American Type Culture Collection
34	?	
35	?	
36	?	
37	?	
38	?	
40	Laryngeal swab ?	J. E. Smith, University of London, London, England
41	Distemper, lung	
42	?	
43	?	
44	Laryngitis in dog	
45	Distemper, lung	
46	Laryngeal swab, dog	
47	Laryngitis, dog	
48	Rat epizootic	S. Rosen, University of Michigan, Lansing, Michigan
49	Rat epizootic	
50	?	Iowa State Veterinary School, Ames, Iowa
51	?	
52	?	
53	Normal guinea pig	Lois Dickinson, Boots Pure Drug Co., Ltd., Nottingham, England
54	Normal guinea pig	
55	?	
56	?	
57	?	University of Tokyo, Tokyo, Japan
58	?	New York University, New York, New York
59	?	
60	?	Washington State College, Pullman, Washington
63	?	Else Krag Andersen, Statens Seruminstitut, Copenhagen, Denmark

modification of the charcoal agar medium of Mishulow *et al.* (13) namely, 2.5% HIB, 0.35% yeast extract, 1.0% soluble starch, 2.0% agar, and 0.4% activated charcoal.

Phage

The methods used for the study of bacteriophage were adapted from those described by Adams (1).

Crude Preparations

HIB cultures of *B. bronchiseptica* were incubated 4 to 6 hours at 37° C and were examined, both directly and after irradiation, for the presence of phage. Irradiation was effected by placing duplicate 15-ml aliquots of each culture in sterile petri dishes. These plates were rotated for 2 minutes in the dark 25 cm below a Fisher cold quartz ultraviolet lamp (95% emission at 2537 Å). After additional incubation for 1 hour at 37° C and refrigeration overnight, their radiated cultures were passed through molecular membrane filters.* Non-irradiated broth aliquots were similarly filtered. The filtrates were then used for phage experiments.

Detection of Phage

Indicator plates were prepared by mixing 0.1 ml of an 18- to 24-hour HIB culture with 2.5 ml of melted (46° C) 0.7% agar and pouring this uniformly over 15 ml of a relatively dry basal layer of infusion agar. After drying at room temperature for 2 hours, plates were ready for spotting. Each strain of *B. bronchiseptica* was checked against every other strain as a potential phage source. As many as 18 discrete droplets of the crude phage preparations could be placed with capillary pipettes on a single indicator plate. The spotted indicator plates were incubated 16 to 18 hours at room temperature or 37° C, and were then examined for evidence of phage plaques. Lytic areas were more clearly visible on plates held at room temperature.

Phage Purification

Phage plaques in the soft agar were removed with an inoculating loop and emulsified in 2.5 ml of melted 0.7 % agar (46° C). This was then seeded with a phage-sensitive culture, layered upon an infusion agar plate, and held 16 to 18 hours at room temperature. If lysis was confluent, a straight wire needle was plunged into the lytic area and used as the phage inoculum for a new overlay plate. When lysis was not confluent, single discrete plaques were used for transfer inocula. Two or more transfers were used to purify each strain of phage.

Preparation of High-Titer Phage Stocks

The method of Swanstrom and Adams (17) was used to prepare potent phage stocks. From the last plate in the purification series, several plaques or loopsful from areas showing confluent lysis were emulsified in 2 ml of sterile HIB and freed of gross particulate material by centrifugation; ½ ml of the supernate was then incorporated into seeded soft agar overlays on infusion agar plates.

*Millipore Filter Corporation, Watertown 72, Massachusetts, pore size employed throughout was 0.45 μ.

Two plates were prepared for each phage supernate and held 16 to 18 hours at room temperature. Areas of soft agar overlay showing lysis were emulsified in 10 ml of HIB, held at room temperature for 30 minutes, cleared of gross particles by centrifugation, passed through a molecular membrane filter, and stored at 4° C in sterile acid-cleaned screw cap tubes.

Decimal dilutions of phage stocks were made in sterile HIB. A calibrated loop (0.01 ml) was used to spot each phage dilution on the indicator plate. After incubation, as above, the titer of the phage and the critical test dilution (CTD) were ascertained (7).

Determination of Host Range Spectra

To determine the sensitive cultures for each stock phage, indicator plates were spotted with both the high-titer phage preparations and the CTD. The CTD was used to avoid so-called plaque artifacts which result from an inhibition of growth, or lysis from without rather than from the complete lytic cycle. Uniform phage inocula of 0.01 ml were delivered by using graduated 0.1 ml pipettes.

For inoculation of the soft agar, cultures of *B. bronchiseptica* and *B. parapertussis*, incubated at 37° C for 24 and 48 hours, respectively, were grown both in HIB and on charcoal agar slants from which the growth was washed with 1 ml of Verwey's broth (18). One drop from the emulsion or 0.1 ml of the broth produced a uniform layer of bacterial growth in indicator plates. *A. faecalis* overlay inocula were incubated 18 hours in HIB only. *B. pertussis* necessarily was incubated 72 hours on charcoal agar only. Prior to spotting, the latter indicator plates required 8 hours' incubation.

After the spots were absorbed, indicator plates seeded with *B. bronchiseptica* and *A. faecalis* remained at room temperature 18 to 24 hours, while those with *B. parapertussis* and *B. pertussis* were incubated at 37° C for 48 and 72 hours, respectively.

Results

In order to compare the effect of two media, cultures of *B. bronchiseptica* were grown in HIB and on charcoal agar. Essentially identical results in detection of phage occurred using either source of inocula for the indicator plates. Occasional discrepancies were noted, when charcoal agar inocula were used, which appeared to be due to overgrowth by the sensitive indicator strain. Therefore, only the results from cultures grown in HIB have been recorded. High-titer phage preparations as well as CTDs were spotted on indicator plates of all stock strains of *B. bronchiseptica*. Few artifacts were produced by the undiluted phage stocks, but some inconsistencies may have been caused by the release of infective phage from the host lysogen on which the phage was enriched. Hence, only lysis due to spotting with the CTD has been recorded here.

The host range spectra of the various phages on cultures of *B. bronchiseptica* are presented in Table II. Aside from the refractory cultures (21, 23, 27, 29, 32, 33, 35, 37, 52, 58, 59, 60, and 63), the following were found to be quantitatively identical in their sensitivity to the various phages: 41, 42, 43, and 45; 47 and 48; and 55 and 56. Consequently, only the first of each of these

TABLE II

Host range spectra of *Bordetella bronchiseptica* phages*

	Sensitive cultures of *B. bronchiseptica*																											
Phage	4	41	40	55	44	46	51	12	38	50	28	57	3	30	25	47	13	20	49	7	5	22	36	34	53	31	54	24	8	26
25′lg†	2	2	2	3	3	3	3	3	3	3	3	3	3	2	3	2	3	3	3	1	3	1	1	1		1				
30/25	2	2	3	3	3	3	3	3	3	3	3	3	3	1	3	3	3	3	3	3	3	3	3	3						
25′sm†	3	3	3	3	3	3	3	3	3	3	3		2	3	3	3	3	3	3	3	3	1	1							
57/51	3	3	3	3	3	3	3	3	1	3	3		3	1	3	3	3	2	1		3			1						2
51/50	3	3	3	3	3	3	3	3	3	3	3	3	3	1	3	3	3	3	1	2					3					
57/49	2	3	3	3	3	3	3	3	3	3	3		2	1	3		3		3		1	3		2						
48/40	2	2	3	2	2	2	3	3	3	2	3	2	2	3	3	1	3		2											
47/20	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3		3	2											
25/46	2	3	3	3	3	3	3	3	3	3	3	3	3	3		1	3								3					
36/4	3	3	3	2	3	3	3	3	3	3	3	1	1	3	3	1		3	3									3	2	
50/13	2	1	2	2	2	2	2	2	2	2	2	2	1	1	2	1	2	1												
63/45	3	3	1	2	2	1	2	2	1	2	2	2	2	2	2	1					2									
43/20	3	3	3	3	3	3	3	3	3	3	3	3	3	1	3	3		1									3			
44/57	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1		2												
40/42	3	3	1	2	2	2	2	1	1	2	2	2	2	2	2			1						1						
8/42	3	3	1	2	2	1	2	2		2	1	2	1	2	2	1		1									1			
46/45	3	3	1	3	1	1	2	1	1	2	2	2	1	2	2			1												
24/4	3	3	2	2	3	2	3	3	2	3	3	3		3	3	1														
42/28	3	3	2	2	3	3	3	3	3	2	3	3		3	3			3												
13/41	3	3	1	1		1	2	1	2	1	1	2		2	2			1												
24/55	2	2	2	2	2	3	2	2	2			3		2	3	3		3												
60/42			1	1	1	1	2	2	1	2		2	1	1	1	1									1					
58/38	3	3	3	2	3	1	2	3	3		3			2	3	3														
32/46	1	3	3	3	3	3		1		3		3	2				3													
25/46	1	3	3	3	3	3		2		3		3	2				3													
21/48	2		1				3	2		2	2			1	2		2	3	3											
49′	3		1				1	1	2						2				3											

TABLE II—*Concluded*

Host range spectra of *Bordetella bronchiseptica* phages*

	Sensitive cultures of *B. bronchiseptica*																												
Phage	4	41	40	55	44	46	51	12	38	50	28	57	3	30	25	47	13	20	49	7	5	22	36	34	53	31	54	24	8	26
59/49							2	1			1				2				3										
53/41	3	3		2			1							1			1		1										
33/57	1	2						1	2		1			2	1														
49/45	3	3										1							1										
51/49															1		1		3										
23/42	2	2	2														1												
41/42	3	2				1			1																				
4/45	3	3										1																	
52/45	3	3											2																
22/42	2	2			1																								
7/42‡	2	2																											
20/42		3																											

*Legend: 1 = less than 10 plaques per spot; 2 = 10 plaques to semiconfluent lysis; 3 = confluent lysis; blank spaces indicate no phage activity.
†lg = large, sm = small.
‡Phages 7/42, 35/42, and 27/42 have identical spectra, and are represented in the table by 7/42.

cultures is enumerated in the table. The phages are designated by the culture filtrate from which they were obtained. This stock number is placed above the culture number upon which they were grown in the purification and enrichment procedure. The designation of prime (′) is used to indicate a spontaneous virulent phage mutant appearing in the stock culture. It was purified and enriched on the same culture in which it appeared. The plaques formed by these phages of *B. bronchiseptica* varied in size from approximately 0.3 to 1.0 mm in diameter. Examples of these are shown in Figs. 1 and 2. Crude phage preparations from ultraviolet irradiated, as well as nonirradiated broth cultures, presented the same spectra on indicator plates. Titration of the crude phage supernates of several strains of *B. bronchiseptica* were made after intervals of 4, 5, and 6 hours' incubation at 37° C. No significant increase in phage titer occurred after these intervals or following postirradiation incubation periods of ½, 1, or 2 hours.

In initial titrations of the concentrated phage stocks, plaques were obtained using 10^{-8} to 10^{-10} dilutions. The CTD was determined for each phage and ranged from 10^{-2} to 10^{-5} of the original concentration. Phage stocks kept at 4° C retained their potency for a few months only. Preservation of refrigerated stock phages was not improved by varying the pH of the HIB from 6.8 to 7.6. Stocks stored in 50% glycerol at −15° C fell in titer more rapidly than those held at 4° C in sterile broth. Phage stocks did not survive freezing in sterile broth. Although bactericidal amounts of thymol had no deleterious effect on viability of the phage stock in sterile broth, a rapid drop in phage titer occurred in the presence of the bacterial host, presumably because of adsorption of phage onto bacterial debris.

TABLE III

Cultures of *Bordetella parapertussis* sensitive to *B. bronchiseptica* bacteriophage*

	Cultures of *B. parapertussis*								
Phage	94-6	94-5	94-7	94-8	94-10	94-41	94-1	94-4	94-3
53/41	3	3	3	3	3	3	3	3	3
41/42	3	3	3	3	3	3	3	3	3
50/13	3	3	3	3	3	3	3	3	3
36/4	3	3	3	3	3	3	3	3	3
21/48	3	3	3	3	3	3	3	3	3
24/55	3	3	3	3	3	3	3	3	3
30/25	3	3	3	3	3	3	3	3	3
25′lg	3	3	3	3	3	3	3	3	3
25′sm	3	3	3	3	3	3	3	3	3
36/42	1	2	1	1	2	1	1	2	2
51/50	1	3	3	3	3	3	1	3	
24/4	3	3		3	3	3	3		3
57/51	3	3	2	3		1		3	
57/49	2	3	3	3	3	3			
8/42	3	2	2		2				
40/42	2	2		2	2				
63/45			2	2					2
58/38	3				2				

*Legend: 1 = less than 10 plaques per spot; 2 = 10 plaques to semiconfluent lysis; 3 = confluent lysis; blank spaces indicate no phage activity.

The close serological relationship of the three species of *Bordetella* suggested that the phages of *B. bronchiseptica* might lyse other members of this genus. Table III indicates the positive results from an experiment in which the CTD of each phage was spotted on indicator plates of nine strains of *B. parapertussis*. In each case these plaques were transferable and examples of the resultant overlay plates are shown in Figs. 3 and 4. All the phage stocks were also spotted, with negative results, on indicator plates containing 51 strains of *B. pertussis*, 7 strains of *A. faecalis*, and several strains of each of the three *Brucella* species. Although faded areas suggestive of lysis appeared on several of the *B. pertussis* plates where phage 29/42 and 51/50 had been placed, these probably were artifacts since they could not be transferred.

Discussion

Lwoff *et al.* (12) in 1950 discovered that ultraviolet (U.V.) irradiation induced prophages to emerge from their symbiotic state within lysogenic bacteria. The prophages apparently acquire virulence, and by lysis of the host are released. A culture of such an inducible lysogenic organism, after exposure, must be incubated to permit residual growth. Active development of phage occurs, after which as many as 90% of the bacteria in the culture may burst and liberate free infective phage particles (4). However, other lysogenic systems have been found to be only partly inducible by U.V., or even completely unaffected (3). Apparently, the phages of *B. bronchiseptica* are in this latter category.

Salient features of this new phage system are the simplicity of obtaining lytic filtrates and the ease of handling and ready growth of the bacterial host. Opposed to these advantages, if phage typing is to have practical application with this species, is the relative instability of the phage preparations and insensitivity of some strains (only 35, or 73% of 48 strains of *B. bronchiseptica* were lysed by the 41 phages). Our examination of the phages suggested that satisfactory preparations could be obtained and held under optimal conditions of storage. Furthermore, it should be noted that this is the first extensive study of this phage system, and a search for lysogens among additional strains might well provide a battery of phages with which a complete typing scheme could be established.

Despite the fact that many of the phages appeared from their host ranges to be closely related, a unique phage pattern was obtained for each strain of *B. bronchiseptica*. No conclusion can be drawn regarding correlations between phage pattern and host range since no information was available on the hosts from which many of these strains were isolated. Nevertheless, it may be noted that the two strains (5 and 22) isolated from man, and the four (8, 24, 53 and 54) from guinea pigs, were characterized by quite limited sensitivity to the 41 phages.

The ability of *B. bronchiseptica* phages to lyse cultures of *B. parapertussis* suggests the presence of similar, if not identical, receptor sites on these bacteria. This lytic cross reaction, in addition to all the other basic similarities that have been enumerated for these species, adds substantially to the justification for their inclusion together in the newly established genus *Bordetella*.

PLATE I

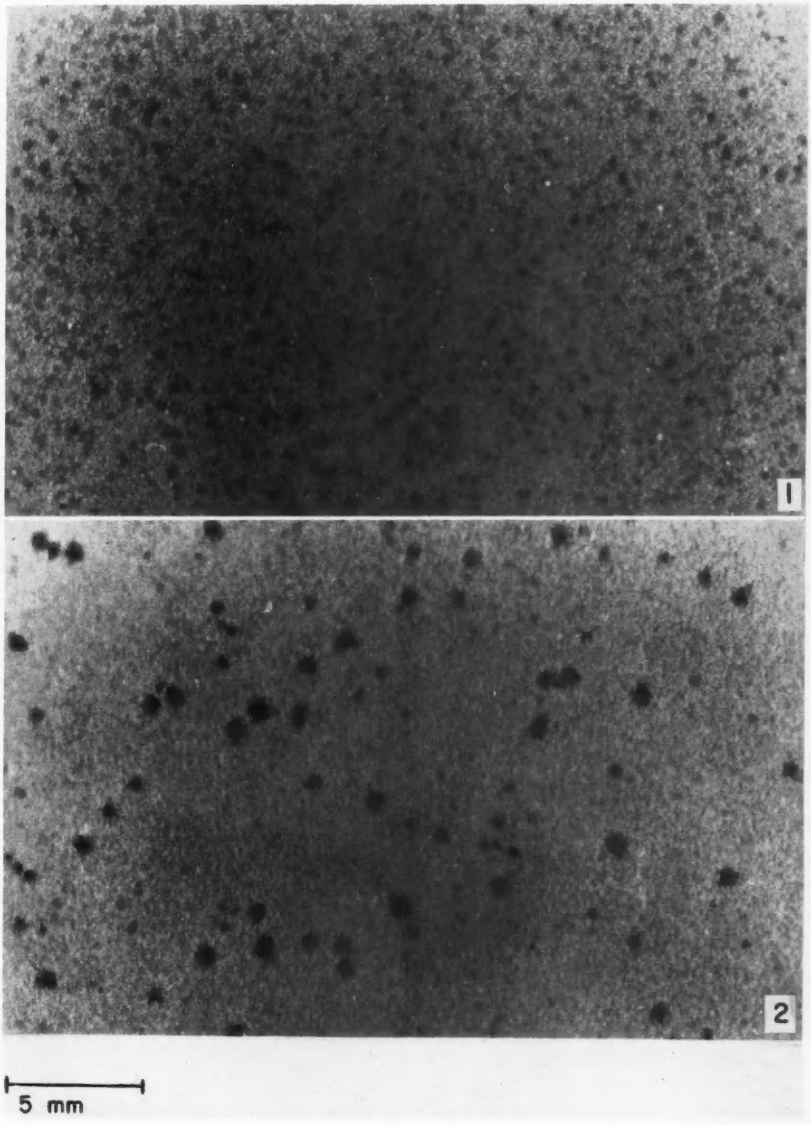

FIG. 1. Phage 7/42 produces small plaques on *Bordetella bronchiseptica*.
FIG. 2. Phage 22/42 produces large plaques on *Bordetella bronchiseptica*.

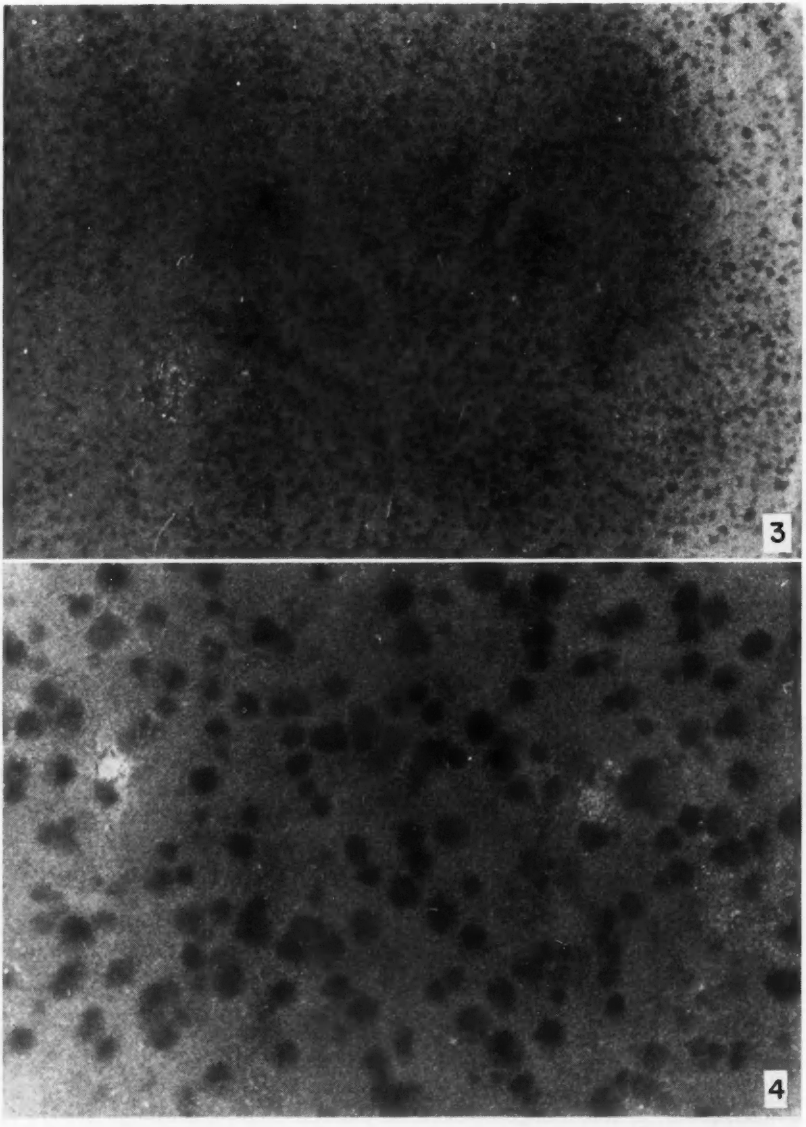

FIG. 3. Plaques produced by phage 51/50 on *Bordetella parapertussis*, culture 94-6.
FIG. 4. Plaques produced by phage 51/50 on *Bordetella parapertussis*, culture 94-10.

Acknowledgments

Grateful acknowledgment is made to Theodore Wallace for collection of many of the *Bordetella bronchiseptica* strains.

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PREPARATION OF NON-INFECTIVE SOLUBLE ANTIGENS WITH GAMMA RADIATION[1]

JOHN R. POLLEY

Abstract

The use of gamma radiation from a cobalt-60 cell for the preparation of non-infective diagnostic antigens for influenza A, influenza B, mumps, smallpox, and herpes simplex has been investigated. It was found possible to destroy the infectivity while retaining most of the complement-fixing activity of all these antigens. The degree of purity of the antigen had no apparent effect on the rate of inactivation, as is the case when formaldehyde is used. Under the experimental conditions described, the degree of inactivation depended on the total amount of radiation applied and not on the dose rate. The kinetics of virus inactivation make it possible to calculate the amount of radiation required to destroy infectivity completely and yet retain most of the antigenicity. If necessary it is possible to apply an additional calculated amount of radiation to destroy residual infectivity without causing loss of antigenicity. Gamma radiation appears to be superior to formaldehyde treatment for the preparation of the herpes simplex antigen which is particularly sensitive to heat and to formaldehyde.

Introduction

Antigens for the laboratory diagnosis of influenza, mumps, herpes simplex, and smallpox are prepared routinely by this laboratory in a stable non-infective form by using formaldehyde (6). With this method it was found to be more difficult to destroy the infectivity of a soluble antigen than that of a viral antigen. It is possible that the soluble antigen may afford protection to the virus particle mechanically and by side reactions consuming some of the formaldehyde. Virus inactivation by means of gamma radiation might be less subject to these difficulties. Ionizing radiation has been studied lately for its effect on bacteria (2) and viruses (1, 3, 9) and has been applied to the preservation of food (5). An ionizing radiation can produce inactivation both by direct action and by the production of toxic agents such as peroxides in the medium (4). In the first phase of this work the possibility of using gamma radiation for the inactivation of virus present in the soluble antigens of influenza, mumps, smallpox, and herpes simplex was investigated.

Materials and Methods

The soluble antigens were prepared by macerating the chorioallantoic membranes from specifically infected chick embryos with one volume of physiological saline in a Waring blendor. This mixture was centrifuged and the supernatant fluid was decanted and placed at 4° C for 3–4 days. The precipitate which appeared on standing was removed by centrifuging at low speed. The supernatant fluid (referred to later as the crude antigen) was then centrifuged at 20,000 r.p.m. (36,000 *g*) to increase the specificity of the diagnostic antigen (7); with the soluble antigen of herpes simplex a centrifugation of only 10,000 r.p.m. (9000 *g*) was used (8).

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Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

The irradiation of samples was carried out at the Commercial Products Division of Atomic Energy of Canada, Ottawa, using a commercially available cobalt-60 cell. For irradiation, 10-ml samples of antigen were placed in small glass vials with bakelite screw caps. These vials were placed in a carrier in the gamma cell. Vials were removed after various periods of exposure to radiation and the antigen was then tested for infectivity, for specific antigenicity in the complement-fixation test, and for stability on lyophilization and storage, as described previously (6). The gamma cell used in these experiments emitted radiation at a dosage of 1.254×10^6 rads/hour (the rad is calculated from the absorption of energy per unit mass and 1 rad = 100 ergs/g). Since the rate of emission of radiation is virtually constant, the dosage administered is proportional to the time of exposure.

Results and Discussion

In the first experiment the effect of various doses of radiation on the antigenicity and infectivity of crude and diagnostic antigens was determined. Samples of a soluble antigen of influenza A, in both the crude and purified form, were subjected to various doses of gamma radiation, as described above. After irradiation, the infectivity titer of each sample was determined after storage for 2–3 days at 4° C. The results are shown in Fig. 1. In addition, the effect of dilution of the antigen was determined by irradiating the purified antigen and portions of it diluted 1:4 and 1:16 respectively. The results are shown in Table I. These experiments were repeated using the soluble antigens of mumps, vaccinia, and influenza B.

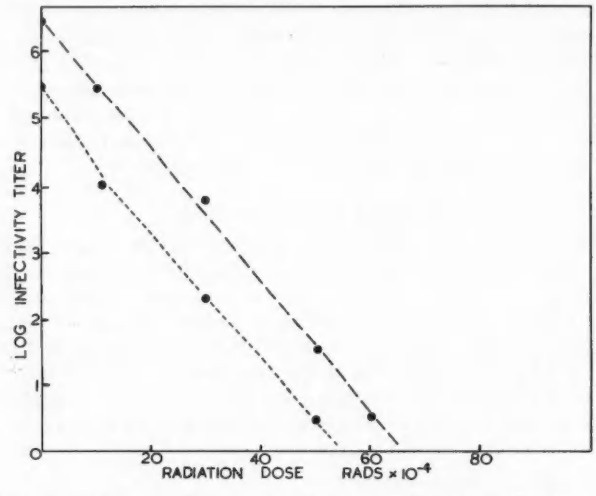

FIG. 1. Effect of gamma radiation on the infectivity titer of crude and purified soluble antigens of influenza A.
●— —● crude antigen. ●- - - - - - -● purified antigen.

TABLE I

Effect of gamma irradiation on various dilutions of soluble antigen of influenza A

Radiation dose (rads)	Undiluted antigen		Antigen 1:4		Antigen 1:16	
	CF titer	Inf.*	CF titer	Inf.*	CF titer	Inf.*
0 (Control)	64	+	32	+	8	+
250,000	64	+	32	+	8	−
500,000	64	+	32	−	4	−
1,000,000	64	−	16	−	2	−
2,000,000	32	−	8	−	<2	−
6,000,000	16	−	<2	−	<2	−

*Inf. = Infectivity.

It can be seen from Fig. 1 that the rate of inactivation by gamma radiation has not been influenced significantly by purification of the soluble antigen of influenza A using high-speed centrifugation. This is in marked contrast to the result observed when formaldehyde is used as the inactivating agent, in which case the presence of additional tissue substances lowers the rate of inactivation. In this study, the time required for complete virus inactivation is related to the initial infectivity titer. As would be expected, therefore, dilution of the antigen influenced the time needed for complete virus inactivation, as shown in Table I. As the antigen was diluted the inactivation time also decreased. The complement-fixing component of the soluble antigen was also destroyed more rapidly as the antigen was diluted. When these experiments were repeated with the soluble antigens of mumps and vaccinia, similar results were obtained, except that these diluted antigens showed a smaller loss of antigenicity. However, the diluted antigens of influenza B and herpes simplex appeared to be more labile.

It remained to be determined whether the degree of inactivation produced by a given dose of radiation was affected by the dose rate. To ascertain whether there was a dose-rate effect, samples of antigens of influenza A and influenza B were irradiated with various doses as before except that one series of antigens was irradiated at a rate of 1.254×10^6 rads/hour while the other series was irradiated at a rate of 6×10^3 rads/hour. Thus, the total amount of radiation received by each series was the same but the rate of administration was greatly different. The results are shown in Table II.

From Table II it can be seen that the rate of inactivation of the virus in soluble antigens of influenza A and influenza B has shown no significant dependence on the rate of administration of the radiation. Within the limits of experimental error of these procedures, a given dose of radiation, whether applied at a high or low rate, has produced approximately the same amount of virus inactivation. This finding is of considerable practical importance because it indicates that a given dose of gamma radiation can be recommended with the expectation that the same result will be attained with gamma-radiation sources of widely varied output. It has a further important application also for it implies, and has been proved, that if a given dose of radiation has been insufficient to produce complete virus inactivation, the material can be subjected to a further dose, the amount of which can be exactly calculated.

TABLE II

Effect of rate of irradiation on infectivity titer of antigens of influenza A and influenza B

Total radiation received (rads)	Influenza A		Influenza B	
	High-dose rate	Low-dose rate	High-dose rate	Low-dose rate
0 (Control)	$10^{-5.5}$	$10^{-5.5}$	$10^{-4.7}$	$10^{-4.7}$
100,000	$10^{-3.0}$	$10^{-2.7}$	$10^{-2.8}$	$10^{-3.0}$
200,000	$10^{-2.0}$	$10^{-1.7}$	$10^{-1.5}$	$10^{-1.5}$
400,000	$10^{-0.5}$	$10^{-0.5}$	$10^{-0.5}$	0

The effect of chemical agents, e.g. formaldehyde, cannot be predicted with such confidence.

To confirm the possibility that non-infective diagnostic antigens can be prepared routinely using gamma radiation, four samples each of antigens of influenza A, influenza B, mumps, and herpes simplex were prepared as described above. Each of these antigens was irradiated as before. After radiation, samples of each antigen were lyophilized, 0.5 cc per 3 cc glass ampoule, using an Edwards centrifugal freeze-dryer. The dried antigens were reconstituted to their original volume with distilled water and tested for infectivity and specific antigenicity as above. The results of a typical experiment are shown in Table III.

TABLE III

Effect of gamma radiation on various soluble antigens

Radiation (rads)	Influenza A		Influenza B		Herpes simplex	
	CF titer	Inf.*	CF titer	Inf.*	CF titer	Inf.*
0 (Control)	64	+	32	+	32	+
250,000	64	+	32	+	32	+
500,000	64	±	32	−	32	−
1,000,000	64	−	16	−	16	−
2,000,000	32	−	16	−	8	−
6,000,000	16	−	8	−	4	−

*Inf. = Infectivity.

From Table III it can be seen that, using gamma radiation, soluble antigens of influenza A, influenza B, and herpes simplex can be rendered non-infective with retention of most of the complement-fixing antigenicity. Similar results were obtained with the soluble antigens of mumps and vaccinia. It appears that a radiation dose of about 1 million rads is sufficient for the antigens prepared as described above. Since the gamma cell used in these experiments had a dose rate of about 1.2×10^6 rads/hour, the total time for destruction of infectivity was less than 1 hour. In a previous study it was found that, using formaldehyde, the diagnostic antigen of herpes simplex was the most difficult to prepare in a non-infective form; the method finally developed involved treatment for 2 days (8). Thus, gamma radiation appears to offer a definite advantage in the preparation of a non-infective antigen for herpes simplex.

Acknowledgment

The assistance of Mr. K. McQueen of the Commercial Products Division of Atomic Energy of Canada, Ottawa, in carrying out the irradiation of the viral samples is gratefully acknowledged.

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LOSS OF IMMUNOGENIC PROPERTIES OF VACCINIA VIRUS INACTIVATED BY FORMALDEHYDE[1]

C. R. AMIES

Abstract

In the experiments here reported, vaccinia virus in the form of partly purified elementary body suspensions was exposed to 0.006 *M* formaldehyde in the presence of 0.02 *M* glycine at 20–24° C. Samples were taken at intervals to determine the rate of inactivation. Assays were carried out by intracutaneous inoculation of rabbits and by the plaque count method in monkey kidney cell monolayers. In addition, the virus suspensions so treated were injected into rabbits and the resulting immune response was studied by serum–virus neutralization tests performed on the rabbit skin and in tissue cultures.

It was found that the rate of inactivation by HCHO decreased with time in accordance with the usual dynamics of disinfection. Similarly, the immunogenic properties decreased in proportion to the fall in infectivity. Minimal quantities of virus could still be detected after 150–250 hours; and samples removed at these times were capable of producing a feeble immune response. Later samples were neither infective nor immunogenic. These experiments lend no support to the belief that HCHO-inactivated vaccinia virus could be employed as a substitute for living virus in immunization against smallpox.

Introduction

At the present time opinions are sharply divided about the value of completely inactivated virus vaccines. Much of this controversy is now centered on the relative merits of "killed" and living attenuated poliovirus vaccines, but outside this area of research there are similar problems of equal magnitude. To what extent, for example, are formalinized influenza virus vaccines effective? No clear answer to this question can yet be given in spite of the extensive studies that were carried out during the pandemic of Asian A influenza in 1957. In contrast to this uncertainty the position of vaccinia virus as a highly effective living attenuated vaccine is unassailable. Yet even vaccinia has disadvantages which prevent its acceptance as an ideal prophylactic. The incidence of severe reactions following Jennerian vaccination and of the fortunately much less common cases of generalized vaccinia and postvaccinial encephalitis are sufficient to justify a continuing interest in preparations which have been completely inactivated. The problem is to develop a method of destroying the infectivity of the virus without impairing its immunogenic properties. Ramon and his colleagues at the Pasteur Institute have long been active in research on these lines. In 1942, Ramon, Boquet, and Richou (9) published their first paper on the effect of heat and formaldehyde on vaccinia virus. From the results of their experiments they concluded that complete loss of infectivity with no decrease of immunizing power could be obtained in this manner. It is important to note that the French workers have also studied and reported favorably on the immunogenic activity of vaccinia virus which has been partly inactivated with HCHO. The differentiation of *partial* and

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Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

complete inactivation is important, as will be disclosed later. Continuing these studies, Ramon and his co-workers (11) a few years ago reported the successful cultivation of vaccinia virus in tissue cultures of bovine embryonic skin. From this culture virus they hope to prepare a formaldehyde-inactivated vaccine to supersede calf lymph. Experimental and clinical studies to support this thesis have not yet been published.

Meanwhile, much has been learnt about the effect of HCHO on viruses, particularly with respect to poliovirus. Gard, a prominent worker in this field, has discussed this subject in two recent communications (5, 6) to which reference should be made for details of what is still a complicated and, to some extent, controversial subject. There seems to be fairly general agreement that the rate of inactivation of poliovirus (and of several other viruses) by HCHO does not remain constant but decreases continuously in the course of the process: in other words, the reaction is not strictly of the first order. However, the inactivation rate seems to conform to certain empirical formulae proposed by Gard (5).

The present experiments were carried out with relatively pure suspensions of vaccinia elementary bodies and the HCHO concentration was maintained at an approximately constant level. Under the conditions chosen for the tests, the course of inactivation showed wide deviations from that of a first-order reaction and it was not possible to determine the length of time required to reduce the infectivity exactly to zero. The results also showed that the immunogenic properties of the virus are destroyed by the time that infectivity is no longer demonstrable.

Methods

Vaccinia Virus

Of the 11 inactivation experiments undertaken seven were performed with a strain of vaccinia which has been maintained for many years at the Lister Institute, London, England: this is propagated exclusively on rabbit skin. In the four additional experiments the source of virus was the Connaught Laboratories vaccinia strain, which is maintained solely on calf skin. The crude skin pulp was triturated with 0.004 *M* McIlvaine buffer of pH 7.0 and partly purified suspensions of elementary bodies were prepared from this material by Craigie's (2) method of differential centrifugation. A centrifugal force of $4340 \times g$ (angle centrifuge) for 30 minutes was sufficient to deposit the virus. The intermediate clarification stages were carried out at $1000 \times g$, using a swinging bucket type of centrifuge. The dilute McIlvaine buffer must be used as dispersing medium for the elementary bodies. Physiological saline and buffers of equivalent ionic strength cause the virus to flocculate.

Inactivation by HCHO

The method of Neumüller (8) as used by Gard (5) was adopted. A solution containing 0.012 *M* HCHO and 0.04 *M* glycine was mixed with an equal volume of partly purified virus suspension having an infectivity of at least 10^7 ID_{50}/ml. The concentrations were thus reduced to 0.006 *M* HCHO (1 volume of 37% formaldehyde solution in 2055) and 0.02 *M* glycine. Sample tubes containing an equivalent concentration of virus only and of virus with glycine but without HCHO were set up as controls. The reaction mixtures were held at

room temperature (20°–24° C) shielded from light and samples were withdrawn at intervals for testing. As each sample was taken the action of the HCHO was arrested by addition of 2 stoichiometric equivalents of sodium bisulphite. HCHO concentration was determined by the method of Nash (7) as modified by Taylor and Moloney (12).

Titration of Virus

Titration of vaccinia virus on monkey kidney cells, either in roller tubes or by plaque counts, provides a simple and reliable method of assay. Experience of these methods extending over a period of 2 years has fully confirmed the favorable reports published by Cutchins and Warren (3, 4). In this laboratory the plaque count technique is now regarded as the method of choice *provided that the preparation to be assayed has a high potency*; but when there is much inactive virus present as a consequence of physical or chemical action the results are unreliable on account of autointerference. Further reference to this problem will be made later. In the present study the main interest was focussed on vaccinia preparations which had been completely, or almost completely, inactivated. As tissue culture methods could not be relied upon, it was necessary to find some other means of detecting and measuring small quantities of active virus in the presence of considerable amounts of non-infective virus. The search led to the unexpected discovery that the rabbit skin is capable of demonstrating minimal amounts of virus under these conditions. Titration of vaccinia by intradermal inoculation is generally less satisfactory than tissue culture methods on account of variability in response of individual animals; nevertheless, for the special purpose of this present investigation, it provided the information needed.

Immunogenicity Tests

Samples of the virus–HCHO–glycine mixtures were removed at intervals, neutralized with bisulphite, and then used as antigens for injection into rabbits. Groups of six animals were used for each antigen and two or three injections were given subcutaneously with alginate adjuvant (1) at intervals of 1 week. The amount of antigen inoculated was 1.0–2.5 ml of virus suspension containing, before inactivation, 10^7 ID_{50}/ml: this was mixed immediately before injection with an equal volume of 4% sodium alginate. Blood samples were taken before immunization and 1 week after each injection. The sera were assayed for antivaccinia antibody by neutralization tests performed both in vitro and in vivo. In both methods the test dose of virus was 100 ID_{50} units in a volume of 0.1 ml. The serum dilution, also in a volume of 0.1 ml, was mixed with the virus and held at 37° C for 90 minutes. At the end of this period, 0.2 ml volume of the serum–virus mixtures was injected intradermally into rabbits or applied to monkey kidney tissue cultures. These tissue cultures were prepared by dispensing a trypsinized monkey kidney cell suspension into roller tubes and by incubating in a medium consisting of 0.5% bovine lactalbumin in Hanks' balanced salt solution to which had been added normal bovine serum to a concentration of 2.0%. On the 6th day when the cell monolayers were well formed the medium was withdrawn and the serum–virus mixture was added without prior dilution. After incubation for 1 hour at room temperature, 2.0 ml of Synthetic Medium No. 597 was added to each tube and incubation

was continued in a rotating drum. Four days later the tubes were examined microscopically for evidence of specific cytopathogenic changes: these, if present, were easily recognized.

The results of these neutralization tests, whether performed in animals or tissue cultures, were expressed as the number of ID_{50} doses of virus neutralized by 1.0 ml of serum. As the test dose of virus was maintained at 100 ID_{50} and the amount of serum mixed with it could not be greater than 0.1 ml, it follows that 1000 neutralizing units per ml was the smallest value that could be recorded. A lower limit could have been obtained by reducing the test dose of virus to, say, 10 ID_{50}, but this was not considered justifiable because the results of a test of this kind become increasingly irregular as the test dose is decreased.

When the immunization course had been completed the rabbits were subjected to a challenge test with a vaccinia preparation of known infectivity, the virus being introduced both by scarification and by intradermal injection. Further samples of blood were collected 1 week after the challenge.

Results

Inactivation Rates

In six experiments the number of samples tested was sufficient to follow the course of the inactivation process with fair accuracy. The results obtained in three of these experiments are shown in the composite graph, Fig. 1. Each curve represents the best fit to a series of values determined by titration in the rabbit skin. Infective potency (ID_{50}/ml) is plotted on a log scale against time of treatment with HCHO. For the sake of clarity, the actual values plotted are not shown but all were within 0.5 log of the value indicated by the curve. The next graph, Fig. 2, refers to a single experiment. In this case the results of both intradermal tests and tissue culture assays are shown. The two curves are of the same type but the skin test reveals small amounts of live virus in samples which produce no cytopathogenic changes in monkey kidney cell cultures. This is not an isolated instance of this phenomenon but the usual finding. It cannot be explained simply as a difference between the susceptibility of rabbit cells in an intact animal and of monkey cells in a tissue culture system because the results obtained by these two methods only disagree when partly inactivated suspensions are being titrated. With highly active fresh suspensions, the tissue culture method usually gives a slightly higher titer. This difference in titration results is more easily explained as the effect of autointerference. Support for this idea can be obtained by direct experiment, as mentioned later in this report.

It must be emphasized that the skin lesions resulting from injection of these partly inactivated preparations were certainly produced by live virus because the same suspension when mixed with antivaccinial serum caused no lesion when injected intradermally.

With the comparatively rough methods of assay at present available, it is not possible to determine when the last quantum of infectivity disappears: and unless there is some defence mechanism on the part of the host to prevent it a small residuum of active virus may propagate sufficiently to constitute an antigenic stimulus even though it may not produce a clinically recognizable infection.

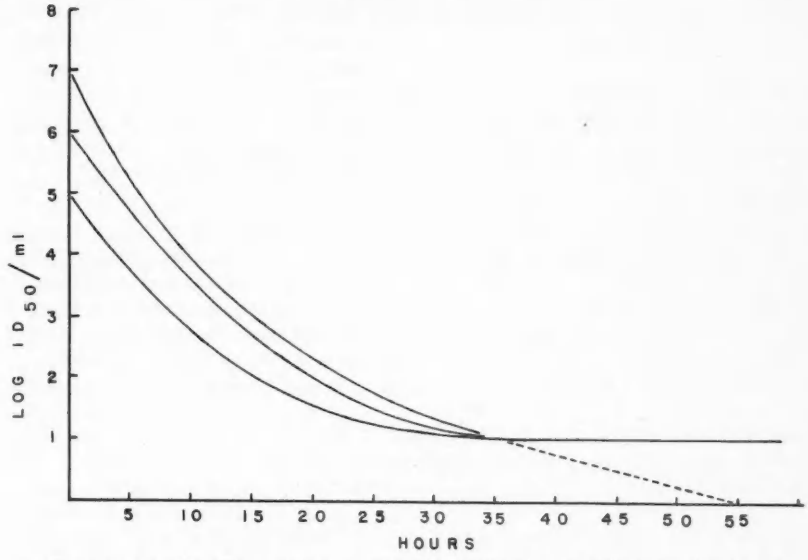

FIG. 1. Inactivation of vaccinia virus by 0.006 *M* HCHO at 20–24° C. Results of three separate experiments, using partly purified elementary body suspensions. Assays performed by tissue culture method.

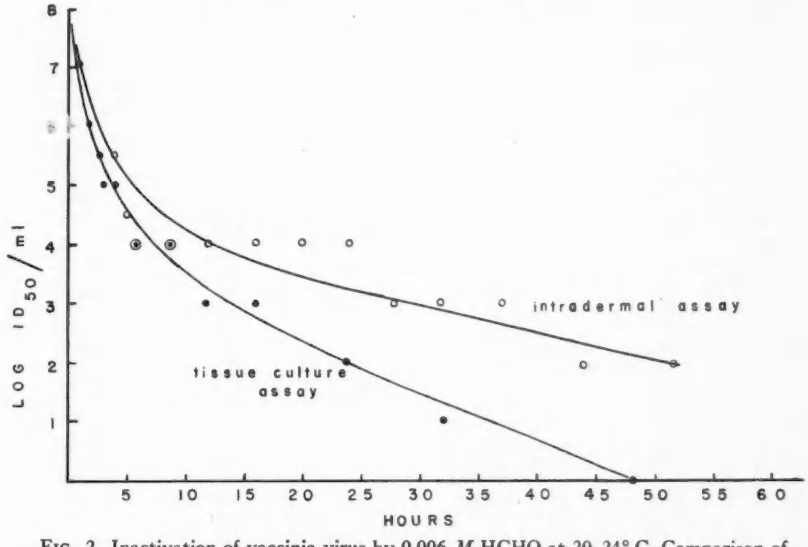

FIG. 2. Inactivation of vaccinia virus by 0.006 *M* HCHO at 20–24° C. Comparison of results obtained by rabbit intradermal method and by tissue culture assay.

Immune Response to Formaldehyde-treated Virus

The immune response to live vaccinia was first studied in order to provide a basis for comparison with the results obtained with HCHO-treated preparations. This was followed by experiments with partly inactivated virus and with suspensions which had just reached the stage of non-infectivity according to available methods of testing. Finally, a few experiments were carried out with virus which had been held in contact with HCHO for longer periods.

Response to Experimental Infection

Rabbits which have been experimentally infected with vaccinia become highly immune. Attempts to reinfect these animals are unsuccessful, even after the lapse of a year or more. This resistance is also reflected in the high antibody content of postinfection samples of serum. The results summarized in Table I are typical examples of immunity acquired in this way. It will be noted that antibody produced as the result of an infection is able to neutralize the virus both in the rabbit skin and in monkey kidney tissue cultures. This is in contrast

TABLE I

Immune response of rabbits experimentally infected with vaccinia virus

Rabbit No.	Infecting strain of virus	Route of inoculation	Number of days after infection	Antibody content of serum: Rabbit intradermal method: number of m.i.d. of virus neutralized by 1.0 ml	Antibody content of serum: Monkey tissue culture method: number of ID_{50} doses of virus neutralized by 1.0 ml
1*a*	Lapine	Scarification	7	<1000	Not tested
			10	1000	Not tested
			14	3200	Not tested
			24	6400	Not tested
1*b*	Calf	Intradermal	5	<1000	<1000
			9	1000	1000
			13	9000	5000
			20	10000	5000
		8 doses s.c.	100	25000	20000
1*c*	Calf	Intradermal	5	<1000	<1000
			9	<1000	
			13	1000	1000
			20	5000	5000
		8 doses s.c.	100	5000	20000
1*d*	Lapine	Intradermal	9	1000	8000
		3 doses s.c.	60	9000	12500
1*e*	Lapine	Intradermal	9	3000	6000
		3 doses s.c.	60	5000	8000
1*f*	Calf	Subcutaneous	9	<1000	5000
			23	3000	25000
1*g*	Calf	Subcutaneous	9	<1000	<1000
			23	3000	5000
1*h*	Calf	Subcutaneous	9	<1000	<1000
			23	1000	25000
1*i*	Calf	Subcutaneous	9	<1000	<1000
			23	3000	5000

NOTE: Rabbits 1*b*, 1*c*, 1*d*, 1*e* were hyperimmunized by repeated subcutaneous inoculations of highly infective virus. s.c. = subcutaneous.

with the results obtained with HCHO-inactivated virus, to be described in a later section.

Response to Partly Inactivated Virus

In one experiment (included in Fig. 1) a virus suspension having an original infectivity of 5×10^7 ID_{50}/ml was exposed to HCHO with glycine for 36 hours at 22° C. At the end of this period 0.1 ml of the suspension produced a vaccinial lesion when inoculated intradermally in rabbits: smaller amounts had no effect. This same suspension was then used to immunize six rabbits, the dose being 1.5 ml mixed with alginate and injected subcutaneously on two occasions 7 days apart. Samples of blood serum were taken before immunization and 7 days after each injection. The titration results presented in Table II show that in each animal the immune response was similar to that produced by inoculation of living virus. The final step in this experiment was a challenge test. On the 25th day following the first injection, each animal was shaved and inoculated percutaneously with the same lapine strain from which the HCHO-treated elementary body suspension was prepared. A highly infective glycerol–buffer suspension was used. None of the rabbits developed more than slight traumatic lesions at the site of scarification whereas two normal rabbits, acting as controls, produced typical vaccinial eruptions.

The results of this experiment can be summarized as follows: The infectivity of the virus was reduced by HCHO treatment from 5×10^7 to approximately 10 ID_{50}/ml. This extremely small amount of active virus, together with the bulk of the virus protein, now completely inactive, produced a solid immunity

TABLE II

Immune response of rabbits to vaccinia virus partly inactivated by formaldehyde

Rabbit No.	Number of days after inoculation	Antibody content of serum: number of ID_{50} doses of test virus neutralized by 1.0 ml	
		Rabbit intracutaneous method	Monkey kidney tissue culture method
2a	7	<1000	<1000
	14	5000	2000
2b	7	5000	9000
	14	10000	9000
2c	7	<1000	3000
	14	5000	9000
2d	7	5000	3000
	14	5000	9000
2e	7	<1000	<1000
	14	5000	2000
2f	7	<1000	<1000
	14	5000	9000

NOTE: Samples of serum obtained before immunization contained no detectable amount of antibody. For further details of this experiment see text.

equal to that acquired as the result of experimental infection by scarification. This finding is in marked contrast with the results described in the next section.

Response to Completely Inactivated Virus

The experiments on inactivation rates, already described, have shown that 0.006 *M* HCHO would usually inactivate vaccinia virus completely within 150 to 200 hours. In one experiment, however, residual infectivity persisted for 250 hours. To ensure complete inactivation, therefore, a period of 300 hours' contact was considered necessary. A full experiment in which this was done will be described.

The virus used for this experiment was a partly purified elementary body suspension prepared from calf skin pulp. This suspension had an infective potency of 10^7 m.i.d./ml when assayed by titration in the rabbit skin. Inactivation was allowed to proceed for 300 hours at 22° C with the usual concentrations of glycine and HCHO. In order to detect residual infectivity four rabbits were inoculated intradermally with 0.2 ml of the inactivated suspension. A control mixture consisting of inactivated virus and antivaccinial serum was also injected into the skin at another site. The resulting skin lesions were trivial and there was no appreciable difference between test and control.

The inactivated material, now known to be non-infective, was then tested for immunogenicity. Blood samples were taken from five normal rabbits: each animal then received 2.5 ml of the inactivated suspension with 2.5 ml of 4% sodium alginate, the mixture being injected subcutaneously. Ten days later a second sample of blood was taken, followed by a second inoculation similar to the first. Blood samples were again taken on the 16th and 30th day from the beginning of immunization. One rabbit died as the result of an intercurrent infection. The remaining four rabbits were then submitted to a challenge test. The hair over the back and flanks was clipped and shaved. An area of skin about 4×4 cm was then lightly scarified and inoculated percutaneously with 0.25 ml of a glycerol–buffer suspension of vaccinia prepared from the same calf elementary body suspension that was used for making the inactivated material. The opposite flank of the rabbit was used for an intradermal titration of the suspension employed for scarification. Two normal animals, acting as controls, were inoculated in a similar manner. In each of these six animals the scarified area developed a typical confluent vaccinial lesion, while the intradermal inoculations showed that the virus used for the challenge had a potency of approximately 10^6 m.i.d./ml. A final blood sample was taken from the immunized rabbits on the 5th day following the challenge.

Five samples of serum from each animal were thus available: these were tested for antibody content by serum–virus neutralization using both intradermal and tissue culture methods. The results are summarized in Table III but comment on these will be deferred until a second immunological experiment using the same HCHO-inactivated suspension has been described.

For this second part of the experiment 15 normal rabbits were divided into three groups of five. Group 1 received 0.75 ml of HCHO-inactivated suspension, group 2 received 0.2 ml, and group 3 received 0.05 ml. In each case the antigen was mixed with 2.0 ml of 2% sodium alginate prior to inoculation by the subcutaneous route. Ten days later a blood sample was taken from each

TABLE III

Immune response of rabbits to vaccinia virus completely inactivated by formaldehyde

Rabbit No.	Number of days after inoculation	Antibody content of serum: number of ID_{50} doses of test virus neutralized by 1.0 ml	
		Rabbit intracutaneous method	Monkey kidney tissue culture method
3a	10	<1000	<1000
	16	<1000	<1000
	30	<1000	3000
	35	3000	6000
3b	10	<1000	<1000
	16	<1000	5000
	30	<1000	6000
	35	1000	15000
3c	10	<1000	<1000
	16	<1000	3000
	30	<1000	5000
	35	1000	15000
3d	10	<1000	<1000
	16	<1000	5000
	30	<1000	2000
	35	1000	45000

NOTE: The animals were challenged by scarification with active virus on the 30th day.

TABLE IV

Immune response of rabbits to vaccinia virus completely inactivated by formaldehyde. Part 2

Rabbit No.	Amount of antigen inoculated, 2 doses (in ml) of:	Antibody content of serum: number of doses of test virus neutralized by 1.0 ml			
		Serum sample taken 10 days after 1st dose of antigen		Serum sample taken 13 days after 2nd dose of antigen	
		Rabbit intracutaneous method, m.i.d./ml	Tissue culture method, ID_{50}/ml	Rabbit intracutaneous method, m.i.d./ml	Tissue culture method, ID_{50}/ml
4a	0.75	None	None	None	15000
4b	0.75	None	None	None	1000
4c	0.75	None	1000	None	20000
4d	0.75	None	1000	None	20000
4e	0.75	None	1000	None	5000
4f	0.20	None	None	None	25000
4g	0.20	None	None	None	15000
4h	0.20	None	None	None	25000
4i	0.20	None	1000	None	25000
	0.20	None	1000	None	5000
4j	0.05	None	1000	None	5000
4k	0.05	None	1000	None	15000
4l	0.05	None	1000	None	10000
4m	0.05	None	1000	None	20000
4n	0.05	None	None	None	5000

NOTE: "None" signifies that 0.1 ml of undiluted serum failed to neutralize 100 m.i.d. of virus.

of the 15 animals and then a second dose of antigen was injected, equal in amount to the first dose. One week after this second dose the animals were again bled and then submitted to a challenge in a manner similar to that described above. The results of this challenge showed that none of the 15 animals possessed any immunity to vaccinia virus. The results of neutralization tests performed on the serum samples taken from the 15 animals are shown in Table IV.

An analysis of the data presented in Tables III and IV together with the information given by the challenge tests leads to the following conclusions:

1. The HCHO-inactivated material, although completely devoid of infectivity, stimulates the production of antibody demonstrable in a neutralization test performed in a monkey kidney tissue culture system. However, antibody cannot be demonstrated when the neutralization test is performed in the rabbit skin.

2. Rabbits which have been immunized with completely inactivated vaccinia suspensions are still fully susceptible to infection by the same strain of vaccinia, in spite of the fact that their sera contain large amounts of antibody demonstrable by serum–virus neutralization tests performed in tissue cultures.

Additional Experiments

Prolonged Treatment with Formaldehyde

In order to complete these studies two experiments were carried out with partly purified vaccinia suspensions which had been in contact with 0.006 *M* HCHO for 20 days at 22° C, a period much in excess of that required to inactivate the virus. These experiments will not be described in detail because the results were uniformly negative. No neutralizing antibody could be demonstrated in the serum of animals inoculated with this material; and when the animals were subsequently challenged with active virus they showed no evidence of immunity.

Higher Concentrations of Formaldehyde

Information on this subject was available in the records of experiments carried out by the writer many years ago at the Lister Institute, London. HCHO was used at a final concentration of 0.1% (0.03 *M*) at room temperature for 18 hours: the reaction was then stopped by adding bisulphite. Rabbits were inoculated with three or more doses of this material after preliminary tests had confirmed that infectivity had been completely destroyed. Serum–virus neutralization tests were performed by the intracutaneous method: tissue culture neutralization methods had not been developed at that time. The results showed unequivocally that these inactivated virus preparations had no immunogenic activity.

The Effect of Autointerference

Vaccinia virus which has been inactivated by HCHO has an inhibitory effect upon the growth of live vaccinia. This example of autointerference is readily studied by blending live virus with inactivated virus in various proportions and titrating the mixtures either intradermally in the rabbit or in tissue cultures of monkey kidney cells. Injection into the skin will detect a smaller amount of live virus than is possible with the tissue culture technique. This is

not the result of a greater susceptibility of rabbit cells in comparison with monkey cells because a potent vaccinia elementary body suspension usually gives a higher end point when titrated in monkey kidney tissue cultures than it gives by the intradermal method. A possible explanation is that the virus can move more freely in the skin and thus has a better chance of encountering a cell which has not been affected by the interfering substance: in a tissue culture monolayer the movement of virus is restricted and the interference phenomenon becomes manifest.

This subject is being studied experimentally and the results will be reported later. At present it is sufficient to state that autointerference is a complicating factor in the titration of preparations which contain much inactive virus. It has added considerably to the difficulties of investigating the rate of inactivation of vaccinia by HCHO or heat.

Discussion

From the results of these experiments it is abundantly clear that HCHO-inactivated vaccinia virus has no value as an immunizing antigen. We can therefore abandon the idea of using this product in place of live virus for the prevention of smallpox.

An interesting and probably important fact emerges from the serum–virus neutralization tests. It was shown that experimentally infected rabbits produce antibodies demonstrable by neutralization tests performed either in the skin or in tissue cultures. On the other hand, animals immunized with HCHO-inactivated virus produced antibody which could be demonstrated in the tissue culture system but not in the skin. The following is a plausible explanation of this difference of behavior. Infective virus is a complete antigen and it therefore stimulates the production of highly reactive (avid) antibody demonstrable by both techniques. Virus which has been inactivated with HCHO is an impaired antigen and it gives rise to a less avid antibody: this is capable of preventing infection of cells in a tissue culture system but not in the skin, where dissociation of an antigen–antibody complex is more readily brought about. This explanation is preferable to assuming that two distinct antibodies are concerned.

By comparing the figures given in Table I with those of Tables III and IV it will be seen that there is not a close correlation between the neutralization titers determined by these two different methods. From this result it follows that the immunogenic potency of a formalinized vaccine cannot be assessed by measuring antibody response in a neutralization test performed in a tissue culture system. The only dependable test is one in which immunization with vaccine is followed by challenge with highly infective virus. In the case of vaccinia it appears that immunity to such a challenge is acquired only as the result of infection with live virus.

Some evidence has been presented that a virus preparation almost completely inactivated by HCHO is an effective antigen. This is in agreement with the statements made by Ramon and his associates (10). It also agrees with the general experience that a subclinical infection with a virus is followed by a high level of immunity. Autointerference may play an important part in this phenomenon. The idea of blending active attenuated and HCHO-inactivated virus to produce a vaccine for human immunization merits further study.

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MALIC ENZYME INDUCTION BY LACTIC ACID BACTERIA

I. A METHOD FOR THE STUDY OF NUTRITIONAL INTERACTIONS[1]

S. J. Deal and Herman C. Lichstein

Abstract

The nutritional requirements for the induction of malic enzyme by washed cellular suspensions of *Lactobacillus arabinosus* 17-5 have been found to be quite similar to the growth requirements for this organism. This similarity permits the use of the measure of rate and extent of malic enzyme synthesis as a tool for studying amino acid interactions and vitamin function.

Introduction

Studies of the nutritional requirements for the growth of bacteria have enabled the accumulation of much information regarding the metabolic role of vitamins, amino acids, purines, pyrimidines, and other growth factors. Since enzyme induction is controlled by nutritional factors, it would appear that a measure of the rate and extent of enzyme induction as influenced by nutrition might afford a rapid method for the study of interactions among growth factors.

Using malic enzyme synthesis by cells of *Lactobacillus arabinosus*, Blanchard *et al.* (3) showed that amino acids and glucose were required for maximum enzyme formation. Vitamins were not required for induction if the bacterial cells were harvested from a medium sufficient in these substances; however, cells grown deficient in biotin or nicotinic acid required these vitamins for optimal enzyme induction.

The present investigation was initiated in order to define further the nutritional requirements for malic enzyme synthesis by washed cell suspensions of *L. arabinosus* and to demonstrate the adequacy of this technique as a suitable tool for studying vitamin and amino acid interactions.

Methods

L. arabinosus 17-5 (ATCC 8014) was grown in Wright–Skeggs medium (17) at 30° C, the bacterial cells harvested by centrifugation in the early to middle logarithmic phase of growth, and washed three times with cold distilled water. Cultures 16–20 hours old were found to possess optimal enzyme forming capacity. However, when vitamin-deficient media were used, longer incubation periods were required to obtain the number of bacterial cells required for induction studies.

The induction of malic enzyme was studied manometrically by conventional Warburg techniques using conditions similar to those employed by Blanchard *et al.* (3). Final concentrations of compounds added to the main compartment of the Warburg flask were: 0.08 *M* phosphate buffer (pH 5.5), 0.08 *M* glucose,

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0.0002 *M* $MgCl_2$, 0.0001 *M* $MnCl_2$, and 0.08 *M* L-malic acid. All materials were adjusted to pH 5.5 before addition to the flasks. To the side arm was added 0.5 ml of bacterial cell suspension containing the equivalent of about 1 mg of dry cells as determined by turbidity measurements in a Klett–Summerson photoelectric colorimeter (420 mμ) and the use of previously standardized curves relating turbidity to dry weight. The final volume in all cases was 3.0 ml. Anaerobic conditions and the incorporation of sodium acetate as used by Blanchard *et al.* (3) were found not to be required and were therefore not employed in the present experiments. The bacterial cells were tipped into the main compartment of the Warburg vessel to initiate induction after an equilibration period of 10–20 minutes.

In agreement with the results of Korkes *et al.* (7), the malic enzyme system in *L. arabinosus* was found to be DPN, rather than TPN, dependent. The absence of a malic dehydrogenase in this organism precludes the possibility that CO_2 was formed via the oxalacetate decarboxylase system. Moreover, malate in the concentrations employed markedly inhibits the oxalacetate decarboxylase enzyme (7). No CO_2 was evolved under aerobic or anaerobic conditions unless malate was added.

Double strength Wright–Skeggs medium (0.2 ml/flask) containing acid-hydrolyzed casein was used as a nitrogen source in some experiments. In many studies, an amino acid mixture based on the growth requirements of *L. arabinosus* (5) was used for this purpose. The individual amino acids were dissolved in distilled water, and an amount added to the Warburg flask to give the following concentrations in micrograms of each L-amino acid: glutamic acid, 400; aspartic acid and alanine, 200; arginine and lysine, 80; cysteine HCl, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine, 40.

Results

Preliminary experiments confirmed the observations of Blanchard *et al.* (3) that substrate, amino acids, and glucose are required for optimal enzyme induction. Slight induction occurred in the absence of added amino acids, probably reflecting the intracellular accumulation of these compounds from the growth medium. Attempts to deplete this pool by several methods resulted in inactivation of the enzyme-forming system.

In order to determine the amino acids required for induction, the bacterial cells were grown in Wright–Skeggs medium and the washed cell suspension was induced in amino acid mixtures using concentrations found optimal for the growth of lactic acid bacteria (5). The extent to which amino acids controlled malic enzyme synthesis was determined by measuring the effect of individual amino acid omission on enzyme production (Table I). It is noted that the effect of single amino acid omission could be measured in terms of the total evolution of CO_2 or the Q_{CO_2} for the final 20-minute reading. Both methods gave similar results. With the exception of cysteine and threonine, those amino acids required for the growth of this organism (10) were also required for optimal enzyme induction. Histidine, which was found to be necessary for induction, is not required for growth. These data suggest that

TABLE I

Effect of omission of individual amino acids from a mixture on malic enzyme induction

Amino acid omitted from a mixture of 18*	Total μl CO_2 evolved in 3 hours	% of 18 amino acids	Q_{CO_2}† for final 20 minutes	% of 18 amino acids
None	845	100	715	100
Glycine	849	100	690	97
L-Alanine	806	95	690	97
L-Proline	854	101	690	97
L-Cysteine	791	94	680	95
L-Threonine	841	99	660	93
L-Aspartic acid	787	93	640	90
L-Lysine	795	94	645	90
L-Serine	737	87	555	77
L-Methionine	638	76	465	65
L-Histidine	507	60	410	58
L-Arginine	413	49	313	44
L-Phenylalanine	394	47	290	40
L-Tryptophan	368	44	279	39
L-Leucine	366	43	272	38
L-Valine	390	46	272	38
L-Isoleucine	281	33	230	32
L-Tyrosine	356	42	230	32
L-Glutamic acid	64	8	28	3

*As described in the text.
†Q_{CO_2} = μl CO_2 produced/mg dry weight per hour; determined for the sixth 20-minute period of incubation.

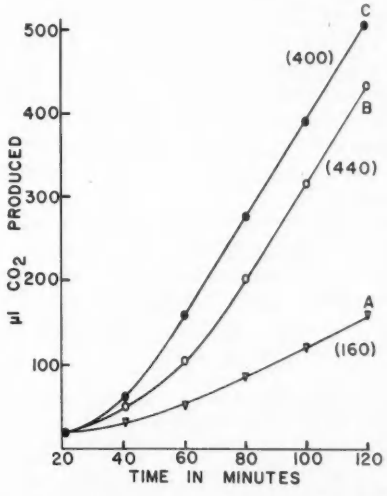

FIG. 1. Replacement of arginine requirement for malic enzyme induction. A = amino acid mixture without arginine, or with and without ornithine; B = amino acid mixture without arginine but with citrulline; C = complete amino acid mixture as described in text with or without citrulline. Concentrations of all amino acids as given in the text. Numbers in parentheses refer to the Q_{CO_2} for the final 20-minute period.

apart from these few exceptions the amino acid requirements for growth and malic enzyme induction are similar.

The observation that the omission of certain amino acids caused a definite decrease in malic enzyme synthesis led to the suggestion that this technique could be used for amino acid replacement studies commonly performed with growing bacterial cultures. The first group studied was the arginine–ornithine–citrulline system. The data in Fig. 1 reveal that citrulline, but not ornithine, was capable of replacing the arginine requirement for malic enzyme synthesis. Neither of these precursors had an effect in the presence of arginine. Repeated experiments consistently showed a lag in induction when citrulline was used, probably resulting from either a permeability barrier to this amino acid or a time requirement for the conversion of citrulline to arginine. Final Q_{CO_2} values indicated that the replacement was complete. These results agree well with those obtained in growth replacement studies (11).

Since D-glutamic acid is capable of replacing L-glutamate for the growth of *L. arabinosus* (1) due to the presence of a racemase (2, 9), it was of interest to study this amino acid in the induction system. When D-glutamate was substituted for the L form in the induction system, a Q_{CO_2} of 285 was obtained as compared with a value of 315 when L-glutamate was used. Omission of glutamic acid, or its attempted replacement with α-ketoglutaric acid and ammonium chloride, resulted in a Q_{CO_2} of only 32.

Some doubt exists concerning the replacement of tryptophan by indole in the growth of this organism. Thus, whereas Snell (15) and Schweigert *et al.* (14) have shown that indole can at least partially replace tryptophan for growth, Rhuland and Bard (13) could find no evidence for the conversion of indole to tryptophan and concluded that growth stimulation occurred in some indirect manner. Data obtained by the induction technique (Table II) indicated that indole could replace partially the tryptophan requirement, but in the presence of tryptophan no enhancement of enzyme synthesis resulted when indole was added. These data agree with those of Snell (15), who showed that indole provided only partial replacement of the tryptophan required for the growth of *L. arabinosus*.

Attention was directed next to some of the B vitamins. Blanchard *et al.* (3) demonstrated that biotin was not directly involved in malic enzyme activity but was probably concerned with stimulating apoenzyme synthesis. Preliminary studies with bacterial cells harvested from Wright–Skeggs medium without added biotin revealed that such cells clumped readily during the

TABLE II

Partial replacement of L-tryptophan by indole for malic enzyme induction

Additions	Q_{CO_2}*
Amino acids†	345
Amino acids + indole	335
Amino acids − L-tryptophan	230
Amino acids − L-tryptophan + indole	280

*Q_{CO_2} calculated for the sixth 20-minute period of induction.
†A mixture of 18 amino acids as described in the text.

washing procedure and possessed little capacity for enzyme synthesis. A comparable clumping effect has been reported previously with this organism when the cells were harvested from media deficient in vitamin B_6 (16). In order to obtain uniform suspensions of biotin-deficient cells capable of malic enzyme synthesis in the presence of added biotin, the cultures were grown in media containing 10^{-5} μg biotin/ml. Both the rate and extent of malic enzyme induction were stimulated by the addition of biotin to the Warburg flask (Fig. 2). It is of some interest to note that biotin was stimulatory over a logarithmic range of 2–3, which is in agreement with that commonly found when *L. arabinosus* is employed for the microbiological assay of this vitamin. Although double-strength Wright–Skeggs medium (containing vitamins, purines, and pyrimidines in addition to the amino acids) permitted better induction than the amino acid mixture, the degree of biotin stimulation was proportionately the same, indicating that the additional constituents contained in the Wright–Skeggs medium were not sparing biotin.

CO_2 is known to spare biotin for the growth of *L. arabinosus*. However, this aspect could not be tested directly in the induction system since the addition of CO_2 to the Warburg flasks caused the reversal of enzyme activity as anticipated. An experiment was therefore performed comparing the effect of biotin

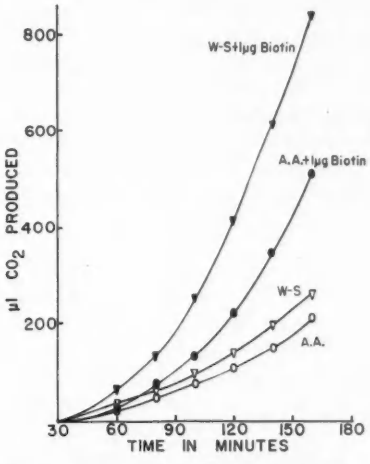

FIG. 2. Influence of nitrogen source on biotin stimulation of malic enzyme induction. AA = mixture of 18 amino acids as described in text; W–S = double strength Wright–Skeggs medium. Concentration as given in the text.

The response of malic enzyme induction to graded concentrations of biotin was as follows:

μg biotin/flask	Q_{CO_2}	μg biotin/flask	Q_{CO_2}
0	320	10^{-3}	610
10^{-5}	360	10^{-2}	750
10^{-4}	400	10^{-1}	800

TABLE III

Effect of growth in CO_2 on biotin stimulation of malic enzyme induction

Condition of cultivation	Growth turbidity*	Extent of induction (Q_{CO_2})†	
		No biotin	+ Biotin (1 μg)
Air	90	155	546
CO_2‡	260	249	726

*Cells grown in Wright–Skeggs medium containing 10^{-3} μg biotin/ml, 24 hours in CO_2 and 36 hours' incubation in air; turbidity read at 640–700 mμ.
†Based on the sixth 20-minute period of induction.
‡Approximately 100%.

on malic enzyme induction of biotin-deficient cells grown in the presence of air and in an atmosphere rich in CO_2. From the data of Table III, it is apparent that the bacterial cells grown in CO_2 required biotin for maximum enzyme induction.

It was considered advisable next to examine the amino acid requirements for malic enzyme induction by biotin-deficient cells. These differed from the requirements of biotin-sufficient cells only in a demand for aspartic acid. Thus, in a typical experiment, bacterial cells grown deficient in biotin could synthesize only 39% of that amount of malic enzyme formed when aspartate was present. The ability of biotin to replace the aspartate requirement is shown by the data plotted in Fig. 3. Moreover, it is manifest that oxybiotin can replace biotin in aspartic acid synthesis. These data are not in agreement with those of Potter and Elvehjem (12), who reported that biotin, but not oxybiotin, could replace the aspartate requirement for the growth of *L. arabinosus*.

Since vitamin B_6 has been demonstrated to be necessary for the synthesis of various amino acids, induction experiments were performed with pyridoxine-deficient cells of *L. arabinosus*. In agreement with growth studies, the amino

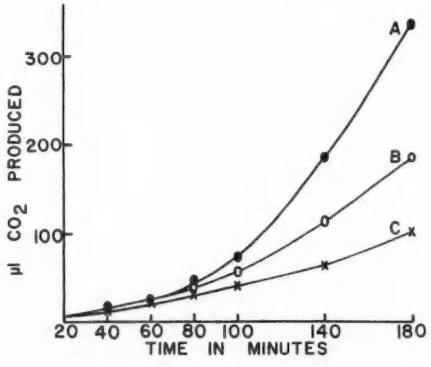

FIG. 3. Replacement of aspartic acid by biotin for malic enzyme induction by biotin-deficient cells. A = amino acid mixture as described in text with or without aspartate but plus 1 μg biotin or oxybiotin; B = amino acid mixture; C = amino acid mixture minus aspartate.

TABLE IV

Effect of omission of individual amino acids from a mixture on malic enzyme induction by vitamin B_6 deficient cells

Amino acid omitted from a mixture of 18*	Q_{CO_2}†	% of 18 amino acids
None	449	100
Glycine	180	84
L-Alanine	222	49
L-Proline	430	99
L-Cysteine	385	86
L-Threonine	131	33
L-Aspartic acid	74	16
L-Lysine	127	31
L-Serine	208	50
L-Methionine	307	76
L-Histidine	180	42
L-Arginine	144	32
L-Phenylalanine	166	37
L-Tryptophan	198	52
L-Leucine	212	55
L-Valine	155	35
L-Isoleucine	131	34
L-Tyrosine	124	31
L-Glutamic acid	77	17

*As described in the text.
†Based on the sixth 20-minute period of induction.

TABLE V

Replacement of certain amino acids by pyridoxal

Amino acid omitted from a mixture of 18*	Q_{CO_2}†	
	No additions	+ Pyridoxal (10 μg)
None	375	425
L-Alanine	180	460
L-Serine	285	355
L-Aspartic acid	250	500
L-Tryptophan	245	270
None	430	500
L-Alanine	250	485
L-Glutamic acid	60	65
L-Lysine	120	300
L-Threonine	290	490

*As described in the text.
†Based on the fifth 20-minute period of induction.

acid requirements for malic enzyme induction by B_6-deficient cells became more exacting (Table IV). A comparison with the amino acid requirements of pyridoxine-sufficient bacterial cells (Table I) reveals that aspartate, lysine, serine, alanine, and threonine were essential for maximum enzyme formation by pyridoxine-deficient cells. Furthermore, these amino acids could be replaced by the addition of pyridoxal to the inducing menstruum (Table V).

Finally, pantothenic acid deficient cells were obtained by growth in Wright–Skeggs medium containing 10^{-2} μg of calcium pantothenate/ml. Such cells exhibited a decreased capacity to induce malic enzyme which was stimulated approximately 30% by the addition of 1 μg of the vitamin to the Warburg flask.

Discussion

The results of nutritional studies on the synthesis of malic enzyme by cells of *L. arabinosus* indicate that induction can be used as a basis for the investigation of the requirement and metabolic functions of vitamins and amino acids. To the extent that these were studied, a close correlation was found between the results using either growth or malic enzyme induction as a parameter.

The amino acid requirements were demonstrated readily by the use of bacterial cells harvested from a complete medium. The deletion of single amino acids from the induction system revealed that those required for optimal enzyme formation were similar to those needed for growth. The specificity of compounds sparing an amino acid requirement would be considered established only if the compound in question has no effect in the presence of a complete amino acid mixture. Experiments which showed the replacement of L-arginine, L-tryptophan, and L-glutamic acid by citrulline, indole, and D-glutamic acid, respectively, fit this criterion.

The importance of vitamins for malic enzyme induction could be demonstrated only when the cells were grown deficient in a specific vitamin. Blanchard *et al.* (3) showed that induction of *L. arabinosus* during growth in media deficient in pantothenate or pyridoxine produced cells with malic enzyme activity equal to that produced in a complete medium. The results of the present study indicate that induction by washed bacterial cell suspensions harvested from media deficient in either of these vitamins was stimulated markedly by the addition of the respective vitamin. The nature of the pantothenate stimulation was not investigated further. None of the vitamins tested in this study exhibited an effect on preformed malic enzyme.

The well-known role of vitamin B_6 in the synthesis of amino acids for the growth of *L. arabinosus* was demonstrated equally well during induction. The importance of this vitamin in the synthesis of alanine, serine, threonine, aspartic acid, and lysine was observed in enzyme formation by cells grown deficient in pyridoxine. The further stimulation of induction by this vitamin probably indicates a role in the biosynthesis of other compounds.

The nature of the biotin stimulation of malic enzyme formation was studied further. Amino acid omission studies revealed that aspartic acid was the only amino acid affected by a biotin deficiency, and complete replacement of this amino acid was achieved by the addition of biotin to the inducing menstruum. These observations are in keeping with the role of biotin in oxalacetate synthesis (8). From growth studies it is known that aspartic acid cannot be replaced by succinate, fumarate, or malate, thus confirming the lack of a malic dehydrogenase in this organism (7). It is probable, therefore, that the addition of biotin permits the synthesis of oxalacetate which can be transaminated to aspartic acid. Further evidence for a transamination reaction is given by the fact that pyridoxal stimulates the formation of aspartate during induction by

vitamin B_6 deficient bacterial cells. Oxybiotin, contrary to results obtained in growth studies (12), could replace the function of biotin in the biosynthesis of aspartate. The fact that biotin stimulates induction even in the presence of aspartic acid suggests that this vitamin is involved in other aspects of apoenzyme synthesis.

Acetate, which is required for the growth of *L. arabinosus* (4), was found unessential for enzyme formation suggesting that its role in fatty acid synthesis is not involved in the induction process.

In conclusion, therefore, enzyme induction by cells of *L. arabinosus* offers a desirable method for the study of metabolic interactions related to protein synthesis. Such a technique is simple and rapid and obviates the need for sterilization of added materials, which is essential for growth studies. Furthermore, pH and temperature can be controlled within very close limits. Recently, Ifland *et al.* (6) have used such a technique successfully in the study of amino acid antagonisms and utilization of peptides and keto analogues of amino acids.

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THE EFFECT OF PLANT ROOTS ON THE NEMATODE POPULATION OF THE SOIL[1]

V. E. HENDERSON[2] AND H. KATZNELSON[3]

Abstract

The number of nematodes in the rhizosphere of wheat, barley, oats, soybeans, and peas is greater than in the adjacent root-free soil. *Pratylenchus* sp. is present in the rhizosphere of grains in greater numbers than in the rhizosphere of legumes; *Paratylenchus* sp. on the other hand showed a preference for legumes. Other genera identified were more abundant in the rhizosphere than in root-free soil, but were not as numerous as *Pratylenchus* or *Paratylenchus*, nor did they show a preference for any of the plants tested. One nematode species (*Tylenchorhynchus*) was present in small numbers and was equally abundant in the rhizosphere and in root-free soil. The consistent distribution pattern of nematodes in the rhizosphere of wheat and of soybeans, observed at high levels of illumination (1200 foot-candles), was not maintained when the illumination was reduced to 300 ft-c or less.

Introduction

Despite the extensive investigations of soil and plant parasitic nematodes, there have been few attempts to determine the influence of normal, healthy roots on the nematode population of soil as has been done for bacteria, fungi, protozoa, and other organisms (4, 10). Where counts of nematodes have been made in the soil surrounding plant roots, the numbers have not been related to those in adjacent root-free soil and thus the extent of the root effect could not be calculated. Nielsen (6) studied the nematode populations of a number of different soil types, both qualitatively and quantitatively, and found that the majority of species were eurytopic, but that some were stenotopic. He recorded the greatest number in rich grassland and the smallest in sand beaches and fallow soil, and estimated that 12% of the species in normal soil fed on plant roots, 33% were bacterial feeders, and 45% algal feeders. Oostenbrink (7) has shown that the populations of a number of species of Tylenchidae vary according to the crop rotations practiced; the figures presented were obtained by analyzing soil samples taken before and after cropping. Oostenbrink (5, 8) has also shown that the populations of certain nematodes, notably some belonging to the genus *Pratylenchus*, are adversely affected by *Tagetes* spp., and he has been able to control populations of these nematodes by growing this plant. Jones (2) in a review of nematode ecology states that "the congregation of soil organisms, including soil nematodes at the root–soil interface is unquestionably due to the supply of organic substances at this point". In the genus *Heterodera*, some species are stimulated to hatch by substances originating at the root–soil interface, the greatest number of larvae hatching from the cysts in root diffusate from the preferred host plant.

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[2]Entomology Research Institute.
[3]Microbiology Research Institute.

Weischer (15) considers that the influence of the root diffusate extends over a short distance of 2 to 3 cm. In addition to stimulating the hatching of larvae of *Heterodera*, root excretions also stimulate molting in species of *Paratylenchus* (9). Henderson (2) has shown that under certain conditions *Ditylenchus destructor* congregates in the rhizosphere of *Trifolium* spp., the greatest number being found in the rhizosphere of plants showing signs of root decay.

Recent studies in the Microbiology Research Institute on the influence of light on microbial activity at the root surface (11) provided an opportunity to conduct exploratory work on the rhizosphere effect on soil nematodes. The results of these studies are presented herein.

Methods

The soil used in all experiments was a light sandy loam. Composite soil samples, each weighing 33 g, were examined. The roots of 15–20 plants of each crop were used to determine the numbers of nematodes in the rhizosphere.

A modified Baermann funnel method was used for extracting nematodes from the soil and rhizosphere samples. In the first two experiments the nematodes were examined alive under a dissecting microscope at magnifications of 80 and 120. In the third experiment semipermanent mounts were made of the nematodes collected, using Seinhorst's (12) method. They were examined under a compound microscope at magnifications of 240 and 540.

After the nematodes were extracted the soil was washed from the roots, oven dried and weighed; the root-free soil was also oven dried and weighed; the calculation of numbers of nematodes was based on the weight of the oven-dried soil.

In the first experiment, wheat, barley, oats, peas, and soybeans were grown in 6-in. pots. Samples were taken after 6 weeks.

In the second experiment, wheat, barley, and soybeans were grown in a tank, 30 in. wide and 16 ft long. One half of the tank was shaded with butter muslin, to reduce the illumination from 1200 ft-c to 300 ft-c. Soil samples were taken at the beginning of the experiment; soil and root samples were taken 4 and 8 weeks later.

Two boxes 18 in. by 12 in. were used for the third experiment. Wheat was planted at one end of each box, soybeans at the other, with 6 in. of bare soil between them. Both boxes were placed in a growth room at 18° C, one box receiving 1200 ft-c for 14 hours daily, and the other 300 ft-c for 10 hours. Soil and root samples were taken 4 months after planting.

Results

The results of the first experiment are presented in Table I. There were greater numbers of nematodes in the rhizosphere of all the plants than in the root-free soil. *Pratylenchus* was most numerous in the rhizosphere of grains and *Paratylenchus* most numerous in the rhizosphere of legumes. The greatest total number of nematodes was found in the rhizosphere of peas, with wheat supporting almost as many.

The results of the second experiment are shown in Table II. Again there were more nematodes in the rhizosphere than in the root-free soil. Under high light

there were greater numbers of *Pratylenchus* on wheat and of *Paratylenchus* on soybeans. Under low light, however, the association of *Pratylenchus* with wheat and *Paratylenchus* with soybeans was not maintained consistently. There is a suggestion of greater numbers of *Pratylenchus* on soybeans and of *Aphelenchus avenae* on both wheat and soybeans under low light than under high light.

TABLE I

Experiment 1. Influence of plant roots on nematode population of soil (numbers per gram oven-dry soil)

	Barley	Oats	Wheat	Soybean	Peas	Root-free soil
Pratylenchus	16.1	34	33	3	19	1.0
Paratylenchus	10.1	10	22	20	87	0.7
Aphelenchus avenae	5.	14	4	3	16	0.1
Total Tylenchida	37.	48	66	29	132	2.4
Total nematodes	275.	135	601	309	708	10.0

Table II also gives the data from the third experiment. There were more nematodes in the rhizosphere than in root-free soil. The total number of nematodes on wheat was again greater under high light than under low light. *Pratylenchus* was most abundant on wheat and *Paratylenchus* on soybean roots. Under low light the relative abundance of these two genera was the same but there were greater numbers of both *Pratylenchus* and *Paratylenchus* on soybeans than on wheat.

TABLE II

Rhizosphere effect on soil nematodes as influenced by light intensity (numbers per gram oven-dry soil)

	High light (1200 ft-c)		Low light (300 ft-c)		Root-free soil
	Wheat	Soybean	Wheat	Soybean	
Experiment 2. Plants sampled Oct. 23, 1959					
Pratylenchus	788	54	52	78	1.0
Paratylenchus	136	554	32	304	0.8
Aphelenchus avenae	8	61	114	73	2.0
Total Tylenchida	936	672	200	454	3.2
Total nematodes	1782	1455	462	1655	11.6
Experiment 2. Plants sampled Nov. 13, 1959					
Pratylenchus	352	87	124	113	0.1
Paratylenchus	58	362	137	38	0.1
Aphelenchus avenae	18	6	27	13	0.7
Total Tylenchida	429	455	289	151	2.1
Total nematodes	1551	788	770	519	20.0
Experiment 3. Plants sampled 4 months after sowing					
Pratylenchus	39	24	48	122	0.2
Paratylenchus	3	123	10	231	0.5
Aphelenchus avenae	28	9	14	40	0.6
Total Tylenchida	78	175	73	393	1.9
Total nematodes	424	281	283	513	10.4

Three other groups of nematodes were identified and counted in these experiments, *Cervidellus* sp., *Dorylaimus* spp., and *Tylenchorhynchus* sp. Comparatively few of these nematodes were found in the rhizosphere and there was no consistent distribution pattern in the rhizosphere of any plant. Of the first two genera more were found in the rhizosphere than in root-free soil, but the increase was small. The number of *Tylenchorhynchus* was approximately the same in the rhizosphere and in root-free soil, suggesting that this nematode was not affected by the roots of either wheat or soybeans. Reduced light did not affect the numbers of any of these genera.

Discussion

Jones (3) indicates that a concentration of nematodes is to be expected in plant rhizospheres. The results of these experiments show that this is indeed the case, although not all the nematodes in the soil are affected to the same degree and some (*Tylenchorhynchus*) may not be affected at all. Other investigators have already reported that the relative abundance of some soil-inhabiting nematodes is influenced by the crop grown (2, 5, 7). Grains appear to favor the increase in populations of *Pratylenchus* (7) and the results of these experiments are in agreement with this observation. On the other hand, *Paratylenchus* increases in the presence of two legumes, soybeans and peas. In the two later experiments in which illumination is supplied to wheat and soybeans at two levels, there appears to be a definite preference of *Pratylenchus* for wheat and of *Paratylenchus* for soybeans at the higher level of illumination, approximately 1200 ft-c or more, preferences which are not maintained at the lower level, approximately 300 ft-c. The over-all effect of decreased illumination (300 ft-c) appears to be a reduction in the number of nematodes, with the exception of moderate increases of *Aphelenchus avenae*, and of *Pratylenchus* on wheat. Other groups of nematodes (*Cervidellus* spp. and *Dorylaimus* spp.) are not affected by the presence of the plant to the same degree nor do they show any particular preference for any of the plants studied in these experiments.

The fact that there are changes in the number of nematodes when the amount of illumination is reduced is of interest. Rouatt and Katznelson (11) hvea examined the bacterial populations on the roots of the wheat in the second experiment. They found that under reduced light there was a marked reduction in the incidence of bacteria requiring amino acids for maximum growth. They suggest that there is both a qualitative and quantitative change in the substances excreted by the roots. Ellenby (1) has recently shown that day length had a marked effect on the reproduction rate of *Heterodera rostochiensis* and Tarjan and Hopper (13) observed similar effects of extended day length on *Meloidogyne incognita*. Wallace (14) has shown that the movement of the potato-cyst nematode (*H. rostochiensis*) is affected by soil type, moisture gradients and the excretions from plant roots. It is quite conceivable that the population growth of *Pratylenchus* and *Paratylenchus* is governed directly or indirectly by the amino acid concentration in the rhizosphere. The inconsistencies observed in the results under low levels of illumination may have been due to the variation in the light regimes under which the plants were growing.

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THE INFLUENCE OF OXYGEN ON THE FORMATION OF NITRATASE IN PSEUDOMONAS DENITRIFICANS[1]

V. B. D. SKERMAN AND I. C. MACRAE

Abstract

Cells of *Pseudomonas denitrificans* which had been grown anaerobically in the presence of nitrate were resuspended in the same medium and grown at constant density with controlled aeration. It was found that whilst an adequate supply of oxygen was maintained no nitratase was formed.

Skerman, Lack, and Millis (4) demonstrated, with cultures of *Pseudomonas denitrificans* growing in a yeast extract peptone medium containing nitrate, that no reduction of the nitrate occurred while there was any detectable oxygen in the solution. The results did not indicate whether this was due to inhibition of the formation of the nitratase or inhibition of the action of the enzyme once formed. In subsequent papers (Skerman and MacRae (5) and (6)) it was demonstrated that, in the presence of adequate oxygen, non-proliferating cells with the preformed enzyme were unable to reduce nitrate although the capacity to do so was not affected during the duration of the experiments. At that stage the second question had not been investigated.

It was shown by van Olden (2) that *Micrococcus denitrificans* cells grown aerobically, with or without nitrate, reduced nitrate anaerobically slowly or not at all. Sacks and Barker (3) noted an essentially similar effect. Allen and van Niel (1) showed that colonies developed when aerobically grown cells of *Pseudomonas stutzeri* were cultured anaerobically in the presence of nitrate, but not in its absence, and stated "that this shows conclusively that each cell of a culture, initially incapable of denitrification, can acquire the ability to do so by adaptive enzyme formation".

This paper reports studies in which cells of *P. denitrificans* adapted to reduce nitrate by growth under anaerobic conditions in nitrate broth have subsequently been grown at constant density in the presence of known concentrations of oxygen and nitrate to observe the effect on the activity of the nitratase.

Materials and Methods

Apparatus

This is shown in Fig. 1. It consists of (i) a 180-ml culture vessel (A) having a No. 3 porosity sintered-glass plate (B); an external water jacket through which water at 28° C was continuously pumped, and an overflow tube (E); (ii) a growth medium reservoir (C) with feed tube (D); (iii) a chilling mixture bath and sample tube (F); (iv) a dropping mercury electrode (d.m.e.) (G); (v) a saturated KCl bridge (H) to a large area saturated calomel reference electrode, and (vi) a perspex shield (J). The electrode assembly and bridge

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Contribution from the Bacteriology Department, University of Queensland, Medical School, Herston Road, Brisbane, Queensland.

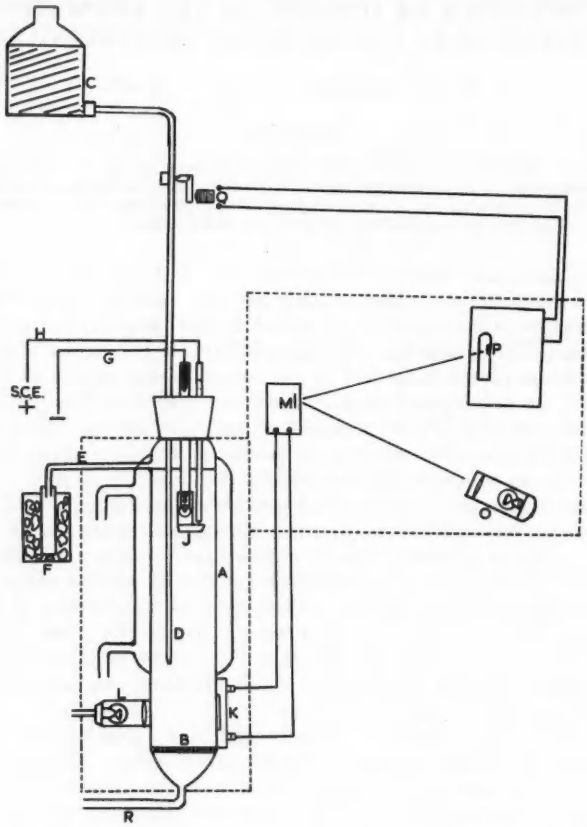

FIG. 1. Sketch of the continuous culture apparatus employed. For explanation, see text.

were identical with that described by Skerman and MacRae (6).

The density was controlled photoelectrically by means of a selenium cell (K) activated by a constant light source (L) and coupled to a sensitive mirror galvanometer (M). A strong light source (O) was fixed so that the angle of incidence allowed the beam of light reflected from the mirror to fall on the sensitive zone of a phototube relay (P) which controlled a solenoid valve (Q) which released fresh medium into the culture vessel. The apparatus was sensitive to the addition of a few drops of culture fluid into 180 ml of culture.

All apparatus shown within the broken lines was encased in light-proof containers. The culture vessel was encased in a plaster of Paris mold which acted as an efficient insulator.

The culture was aerated via tube (R) with 100 ml of gas per minute. The

d.m.e. was coupled to a Cambridge recording polarograph capable of recording for 40 minutes. Photographic papers were changed and the catchment reservoir for mercury emptied at these intervals, without interrupting the gas flow.

Nitrate Estimations

The method described by Skerman and MacRae (5) was used throughout. Only the pure form of zirconyl chloride has been found satisfactory. The nitrate-reducing capacity of a suspension of standardized density was obtained by determining the amount of nitrate reduced anaerobically in a period of 20 minutes. The values quoted in Figs. 3 and 4 are expressed in terms of the change in the diffusion current for nitrate (in millimeters of galvanometer deflection) over this time interval.

Media

The peptone – yeast extract – nitrate medium and method of culture used in previous experiments (Skerman, Lack, and Millis (4)) were again employed.

Procedure

The medium, 162 ml containing 0.2% KNO_3, was placed in the vessel and aerated until a constant oxygen level was obtained. Air at 100 ml per minute was found to be an inadequate oxygen source and an air–oxygen mixture containing 48% oxygen was finally employed. Frothing was prevented by the addition of General Electric Silicone Antifoam 60.

The inoculum was prepared from cultures in the late logarithmic phase. The cells were suspended in a sample of the medium and the density adjusted so that the addition of 18 ml to the culture vessel yielded a final density of 5×10^9 cells per ml. Suspension in phosphate buffers, as in previous experiments, introduced a lag period which was not desirable.

With each addition of fresh medium an equal amount overflowed and was collected in the chilled vessel. The volume of overflow was measured at intervals. The nitrate-reducing capacity was measured initially and at time intervals on equal quantities of the constant density overflow. Continuous oxygen recordings were made during the experiments.

Results

The cumulative overflow volumes plotted against time are shown in Fig. 2; an overflow of 180 ml represents a doubling of the population in that time interval. The nitrate-reducing capacity of the suspensions of constant density collected from the overflow are plotted against the time of sampling in Fig. 3.

In Fig. 4 the nitrate-reducing capacity is plotted in terms of generations of growth. The solid line is the theoretical curve expected if the nitratase ceased to be formed under conditions of adequate aeration, but at the same time did not undergo any other form of degradation. The plotted points indicated by the symbols were derived, for each successive generation, from the experimental curves indicated by similar symbols in Figs. 2 and 3. The oxygen concentration in solution was maintained at well above the critical value (6) throughout the experiments.

In a single experiment in which air was used as the source of oxygen, dis-

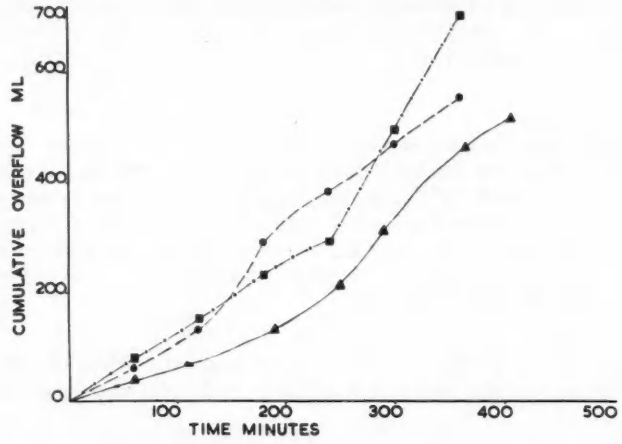

FIG. 2. Growth of *Pseudomonas denitrificans* in yeast extract – nitrate – peptone – water as indicated by the cumulative overflow from a volume of a 180-ml culture grown at constant density under controlled oxygenation. The symbols ▲, ■, and ●, distinguish data obtained from three experiments.

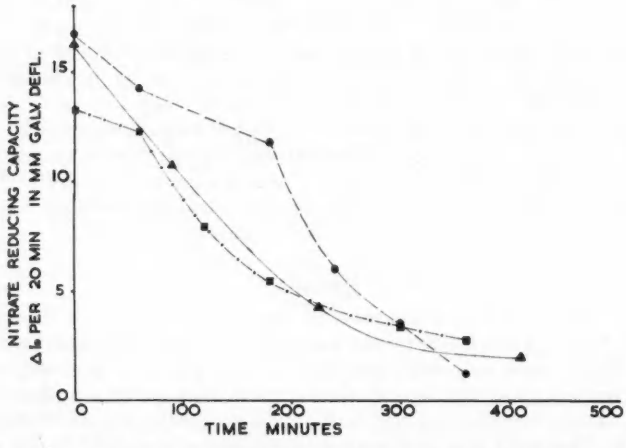

FIG. 3. The change in the nitrate-reducing capacity of samples of the overflow population during the course of the population increase illustrated in Fig. 2 (the symbols correspond). Nitrate-reducing capacity is defined here and in Fig. 4 as the change in the magnitude of the diffusion current (I_d) for nitrate over a 20-minute period and is quoted in millimeters of galvanometer deflection.

solved oxygen could be detected for the first 40 minutes of the experiment only. The population passed through two generations during the whole period of the experiment. A large portion of this population increase occurred

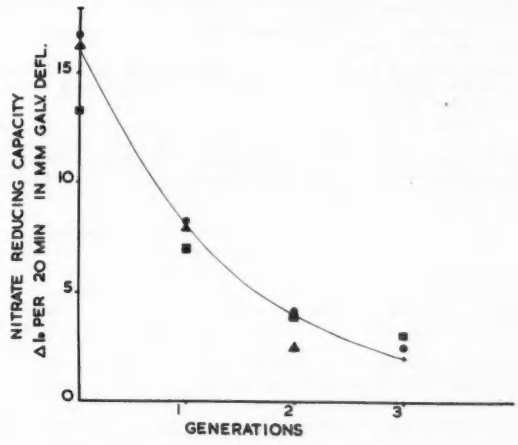

FIG. 4. The changes in nitrate-reducing capacity in relation to generations of cell growth (derived from data illustrated in Figs. 2 and 3). For explanation see text.

during the first 40 minutes while dissolved oxygen was still detectable. By deriving from Fig. 4 the reduction in the concentration of nitratase which would have been associated with this portion of the population increase, it was found that it was approximately equal to the total reduction in nitratase concentration determined over the whole period of the experiment. This indicates that no change occurred in the nitratase concentration *per unit density of cells* during the subsequent period when the oxygen supply was inadequate, despite the continued increase in the total population.

Discussion

The experiments demonstrate that nitratase production ceases when an adequate supply of oxygen is available and that, within the limits of the experimental evidence, this enzyme once formed is not affected by the presence of oxygen. This is in accord with observations made in previous experiments (Skerman and MacRae (6)) in which cells aerated in a buffer mixture showed no detectable change in nitrate-reducing capacity over the time interval employed. The results also support the work of van Olden (2) and others who have studied the formation of the enzyme from small inocula.

The result of the single experiment with air suggests that nitratase continues to be produced by a growing population of cells which is supplied with an amount of oxygen which does not meet the respirational requirements and that it is the partial pressure of oxygen in solution and not merely its availability which determines whether nitratase is produced.

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ENCEPHALITIS IN EXPERIMENTAL COXSACKIE B-3 INFECTION[1]

E. Irene Grodums and George Dempster

Abstract

A study has been made of the vulnerability of various parts of the brain in experimental Coxsackie B-3 virus infection. The standard prototype Coxsackie B-3 virus and a stock of inbred albino mice have been used for this purpose. The sites of greatest susceptibility have been precisely defined by thorough examinations of the whole brain and it has been observed that in 7- to 12-day-old mice the lesions are confined to sensory centers.

Furthermore, observations have been made upon the effects of various factors such as the age at the time of inoculation, the state of myelination in different parts of the brain, and the route of inoculation. The complete resistance of the cerebellum to damage in spite of multiplication of virus in this site is of considerable interest.

In the past little attention has been paid to the anatomical distribution of the brain lesions caused by the Coxsackie group B virus. It would seem to be profitable to direct attention to the features of the experimental disease in view of our lack of basic information regarding many non-fatal illnesses which affect the brain.

Introduction

In an earlier paper (3) a study was made of the age factor in experimental Coxsackie B-3 infection in the mouse. It was revealed that the susceptibility of the heart and the brown fat in the weaned animal was greater than in the sucklings, and even the adult mice were moderately affected. At the same time, the dramatic change in the susceptibility of the brain to damage by Coxsackie B-3 virus during the first 15 days of life was noted. In newborn mice, the brain, with the exception of the cerebellum, was severely damaged and in some instances almost liquefied, yet 15 days after birth intracerebral inoculation of the host produced no visible pathological change despite the fact that the virus might persist in that organ for at least 7 days.

The aims of the present studies were twofold, firstly to establish whether or not lesions did develop after a much longer period of observation in older mice, and secondly to observe and evaluate the vulnerability of the various parts of the brain in Coxsackie B-3 infection. At the same time, it was hoped to determine to what extent this vulnerability to experimental infection depended upon route of inoculation, physiological function, and state of myelination.

Materials and Methods

Mice

The mice used in these investigations were from the same stock as described in the preceding papers (3, 4). Groups of 4-, 7-, 9-, 12-, 15-, 17-, and 63- to 70-day-old infected mice were studied.

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Mice of the same age were pooled and redistributed by stratified random sampling before inoculation. In addition, normal newborn to 17-day-old and 70-day-old mice were used to observe the development of myelin in a total of 90 brains.

Virus

The history of the virus strain used has been described before (3). Mouse carcass suspension was used for inoculation and the titer of this suspension in newborn mice was LD_{50} $10^{-6.42}$ by the intracerebral route and LD_{50} $10^{-6.71}$ by the subcutaneous route. The same material titrated in human kidney tissue culture had a TCD_{50} of $10^{-3.33}$. An inoculum of 0.03 ml of a 1:100 dilution of this suspension in Hanks balanced salt solution was employed in the inoculation of the mice by various routes throughout these experiments. Penicillin (250 units/ml) and streptomycin (250 μg/ml) were added to the inoculum 20 minutes before inoculation.

Histological Examination and the Scoring of Lesions

Histological preparations were carried out as already described (3), except when the brain was examined for the presence of demyelinating lesions in which case the method of Marchi was used (13), in addition to haematoxylin–eosin, Nissl's, and Luxol–Schiff's stains.

The microscopical lesions were scored by marking a brain free of lesions as 0, mild degeneration combined with a scanty perivascular infiltration as +, if also a few necrotic foci were present as ++, extensive rarefication as +++, and ++++ when most of the cerebral cortex had liquefied. Finally, susceptibility was graded on a percentile basis using the formula:

$$\frac{(nx1)+(nx2)+(nx3)+(nx4)}{nx4}$$

when n = the number of mice and $nx4$ = maximum possible score. Percentage expression makes possible comparisons of scores in groups of varying size (7).

The presence of virus in the harvested brain was tested in continuous human kidney cell cultures (3) or in suckling mice. The mice not older than 24 hours were inoculated intracerebrally with 0.03 of a 1:100 dilution of the homogenized brain from the experimental animals and were examined daily for the symptoms of Coxsackie B infection. In doubtful cases the brain and the brown fat were examined histologically.

Results

Does Coxsackie B-3 Virus Cause Lesions in the Brain of 17- and 63- to 70-day-old Mice after a Prolonged Incubation Period?

Ten 17-day-old mice were inoculated intracerebrally and allowed to survive for 28 days. The animals were killed and the complete brain of each animal was sectioned at 8 μ. The examination of every 30th section did not reveal any evidence of demyelination or any other pathological change.

As part of the same study 40 adult, 63- to 70-day-old, mice were inoculated intracerebrally and killed at different time intervals. The brains were examined in the same way as described for the younger animals. Ten mice were killed

after a 7-day interval, a further 10 after 14 days, and the remaining 20 after 28 days. Lesions were not found in the brain of any single individual in this adult group.

Despite the fact that no lesions could be found in these two age groups, the virus could be recovered from the pooled brains of 17-day-old mice up to the 13th day after inoculation and up to the 10th day from the pooled brains of the 63- to 70-day-old mice. These facts were ascertained in a separate experiment in which groups of six animals were killed at daily intervals from 7 to 14 days after inoculation and the pooled brains from each day were tested for the presence of Coxsackie B-3 virus by the inoculation of newborn mice with a 1:100 dilution of the pooled brain suspension.

A Comparison of the Susceptibility of 4- and 12-day-old Brain When Inoculated by the Intracerebral or Subcutaneous Route

Most of the 4-day-old brain inoculated by the intracerebral route had undergone very severe pathological changes: there was a loss of nerve cells, the ground substance showed swelling, and a widespread cyst formation (3). The remaining nerve cells were degenerating or necrotic. Gitter cells appeared to predominate among the cell population. In the majority of cases the cerebrum, for all practical purposes, was completely liquefied. In the other parts of the brain as well as in the older age groups the process appeared to be less destructive. The degenerative features of the pathological changes, however, overshadowed the inflammatory ones.

When compared to the 12-day-old brain, it is clear from Table I that there has been a tremendous decrease in the susceptibility of the brain during the short 8-day period of growth. Furthermore, it can be seen that by contrasting the results of the different routes of inoculation that, whereas the frequency of pathological change remains unaffected by the route of inoculation, there is a definite difference in the severity of the reaction as calculated upon a percentile basis. A severer response was elicited by direct intracerebral inoculation.

TABLE I

Susceptibility of the brain of 4- and 12-day-old mice

Age, days	Killed, days after inoculation	Route of inoculation	Frequency and severity of brain lesions					
			0	+	++	+++	++++	Score %
4	5 to 7	IC	0	0	0	4	4	87
4	5 to 7	SC	0	2	3	2	0	50
12	7	IC	6	2	1	0	0	11
12	7	SC	6	3	0	0	0	8

The Susceptibility of 4-day-old Brain Inoculated by the Cerebellar Route

In all experiments it was noted that the cerebellum remained completely unscathed. This provided an opportunity to determine whether the extreme susceptibility of the 4-day-old cerebrum was in part due to the trauma of inoculation. It proved very simple to make direct inoculation of the brain by the cerebellar route and thus minimize cerebral trauma. There was a

slight diminution of the severity of response when the cerebellar route was used and, despite the insusceptibility of the inoculated area, very extensive damage was recorded in the cerebral tissue (Table II). The distribution of the lesions in the affected parts of the brain did not appear to vary, whichever route was used for the inoculation of the virus, and the cerebellum was not affected even when the cerebellar route of inoculation was used.

TABLE II

Susceptibility of the cerebrum of 4-day-old mice

Killed, days after inoculation	Route of inoculation	Frequency and severity of cerebral lesions					
		0	+	++	+++	++++	Score %
5 to 7	Cerebral	0	0	1	1	8	92
4 to 7	Cerebellar	0	0	4	4	2	70

The Distribution of Lesions in the Brain of 7- to 12-day-old Mice

The younger age groups were not included in these studies since the pathological changes in the brain proved to be too diffuse to observe any selective pattern whatsoever. The general finding in the 7- to 12-day-old mice was that the lesions were not confined to one particular region, but some areas were more commonly and more severely affected than others (Fig. 1). For instance, the most susceptible area in the cerebrum was the hippocampus (Figs. 2 and 3). In the thalamus, it was the lateral, anterior, and ventral nuclei (Fig. 4) which were most commonly affected, while in the midbrain the posterior quadrigeminal body (Fig. 5) and the medial geniculate ganglion

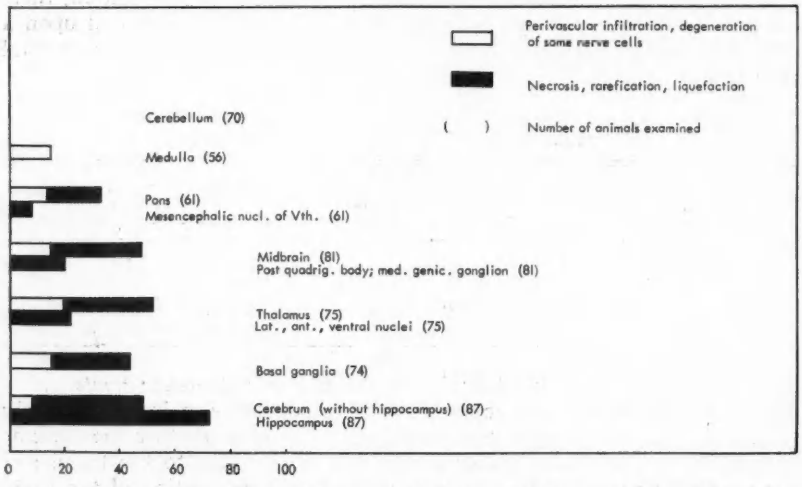


FIG. 1. Distribution of lesions in mouse brain inoculated between the age of 7 and 12 days. Percentage of inoculated animals showing involvement of specific areas.

(Fig. 6) suffered, and in the pons the area of the mesencephalic nucleus of the fifth nerve was often involved (Figs. 7, 8, and 9). The medulla was least affected and the cerebellum not at all. It is notable that all these lesions are confined to sensory centers.

The Development of Myelin in the Brain of Normal Mice and Changing Susceptibility to Coxsackie B-3 Virus

The myelination did not appear to progress in all mice of one age at exactly the same rate. The reported ages are the earliest at which the myelin could be demonstrated in the particular part of the brain.

Myelin could not be demonstrated, at least not by Luxol–Schiff's reaction, before the age of 7 days. In the brain of 7-day-old mice traces of myelin could be found in medulla, pons, and midbrain (brachium pontis). No obvious changes in the degree of myelination could be observed in mice between the ages of 7 and 11 days. At 12 days there was more myelin present in pons, traces of myelin could be found in cerebellum, but none in the cerebrum. A very faint positive reaction to the Luxol stain was present in the cerebra of the 13-day-old mice. Although in the 17-day-old cerebrum part of the white matter stained with Luxol blue, indicating the presence of myelin, most of the fibers were still pink, while the white matter of the adult brain stained true blue throughout.

From these studies it is obvious that myelin is present in all parts of the brain in 17-day-old mice, but myelination is not complete until later.

In Table III an attempt has been made to correlate the extent of the brain lesion with the degree of myelination as established by the Luxol stain. A clear-cut relationship between the two conditions was not apparent, particularly, as the cerebellum remained free of lesions at all ages and at all stages of myelination. It seemed that the susceptibility of the nervous tissue in the midbrain, pons, and medulla decreased as the myelination progressed. The extent and the frequency of the lesions in the cerebrum, on the other hand, seemed to decrease before myelin could be demonstrated.

The Development of Coxsackie B-3 Virus in "Unmyelinated", Myelinating, and Myelinated Brain

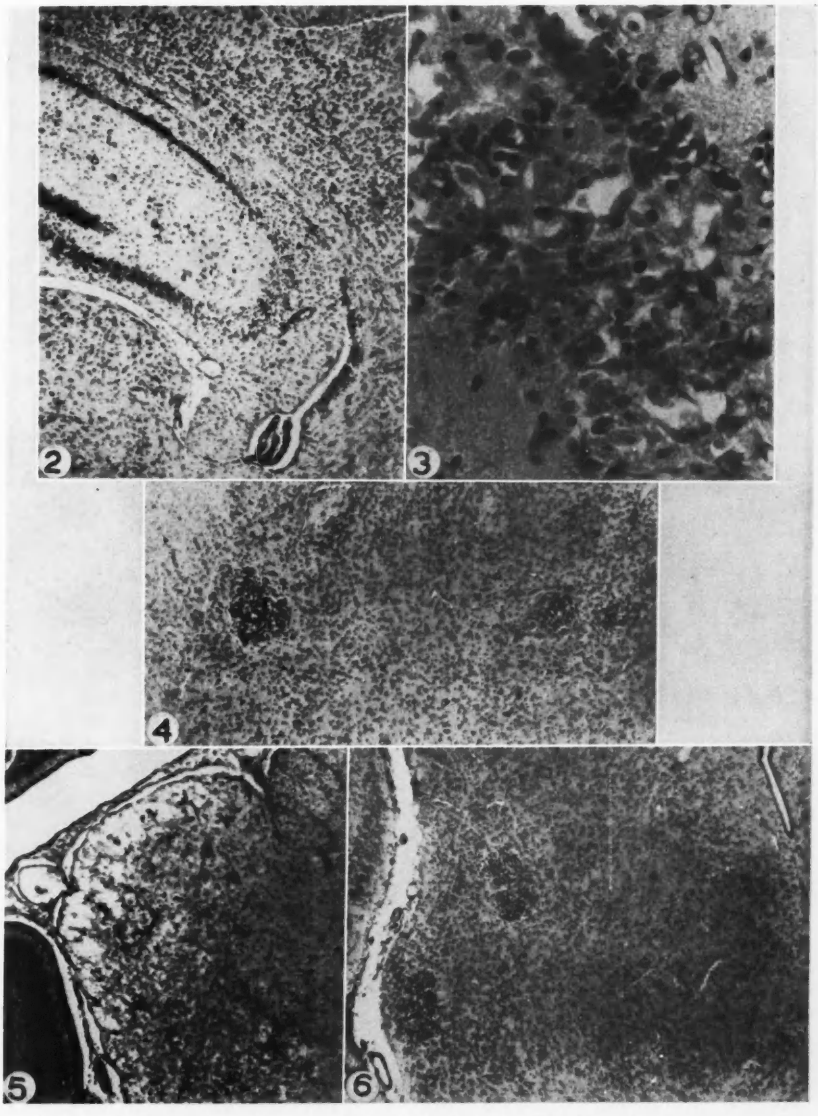
It was decided to investigate the titers of Coxsackie B-3 virus in the major anatomical areas of the brain under conditions in which the state of myelination could be most clearly defined. It was felt that as far as could be determined with the methods at hand the 4-day-old animal was representative of the stage immediately prior to the active laying down of myelin ("unmyelinated"). The 17-day-old animal was one in which the process of myelination was still actively in progress and was midway between the stage of initiation and completion of the myelination process. In the 63- to 70-day-old animal myelination was considered to be complete.

Thus "unmyelinated", myelinating, and myelinated brains were obtained from mice inoculated subcutaneously with the standard inoculum of Coxsackie B-3 virus when 4-, 17-, and 63- to 70-day-old. Four days after inoculation cerebrum, midbrain, cerebellum, and medulla were harvested separately. Each part was washed three times, the corresponding parts were pooled and

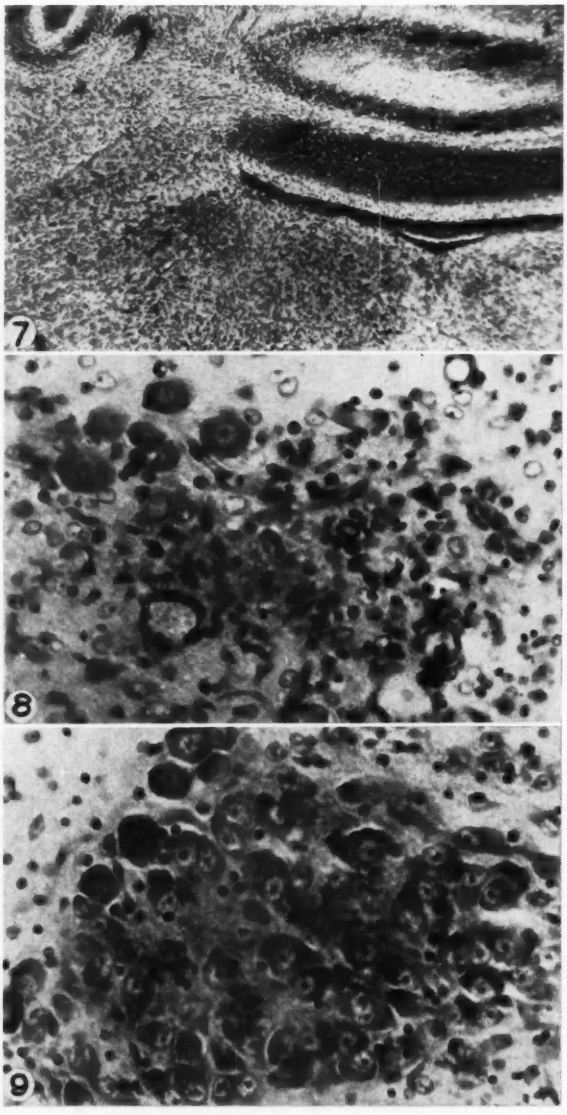
TABLE III

The extent of brain lesions at various stages of myelination

Age, days	Cerebrum and thalamus		Midbrain and pons		Medulla		Cerebellum	
	Lesions	Myelin	Lesions	Myelin	Lesions	Myelin	Lesions	Myelin
4	Extensive, diffuse	None	Extensive, diffuse	None	Mild	None	None	None
7	Less extensive, diffuse	None	Mild, localized	Traces	Decreasing	Traces	None	None
9	Mild to severe, localized	None	Decreasing	Traces	None	Traces	None	None
12	Localized, confined to hippocampus	None	None	Increasing	None	Increasing	None	Traces
15	None	Traces	None	Increasing	None	Increasing	None	Increasing



FIGS. 2–6. Brain of suckling mice inoculated with Coxsackie B-3 virus.
Figs. 2 and 3. Cerebrum—hippocampus, H.E. ×40 and ×294. Fig. 4. Thalamus—thalamic nuclei, H.E. ×40. Fig. 5. Midbrain—the posterior quadrigeminal body, H.E. ×40. Fig. 6. Midbrain—the medial geniculate ganglion and pulvinar, H.E. ×40.



FIGS. 7 and 8. Pons—mesencephalic nucleus of the fifth nerve in a suckling mouse inoculated with Coxsackie B-3 virus, H.E. ×40 and ×294.

FIG. 9. The mesencephalic nucleus of the fifth nerve in normal suckling mouse, H.E. ×294.

ground, and the 20% suspensions were titrated twice on continuous human kidney cell cultures. Four cultures were inoculated with each dilution. The TCD_{50} has been calculated according to Kärber's method (Table IV).

These results do not indicate that the virus would reach a significantly higher titer in any particular part of the brain when inoculated into 4-day-old mice. In older mice (17- and 70-day-old), the virus had not multiplied enough during the 4 days after inoculation to show any titer in human kidney cell cultures.

TABLE IV

TCD_{50} of Coxsackie B-3 in the brain of mouse 4 days after subcutaneous inoculation

Age, days	Cerebrum	Midbrain	Cerebellum	Medulla
4	$10^{-2.5}$	$10^{-2.0}$	$10^{-2.25}$	$10^{-2.50}$
17	0	0	0	0
63 to 70	$<10^{-1}$	0	0	$<10^{-1}$

It is notable that, despite the fact that lesions were never observed in the cerebellum, the virus was found to have reached as high a titer in this area as in those parts of the brain which were severely damaged.

Even from the cerebrum of the 4-day-old mice, the virus titer was very low and this suggested that the human kidney cell cultures might not be a sensitive enough medium to reveal virus in the less susceptible older mice or to show variations among the titers in the various parts of brain. Another test, therefore, was carried out during which the tissue suspensions of the infected brain were tested in newborn and 1-day-old mice. The brain of the 4-, 7-, 17-, and 63- to 70-day-old mice were harvested again on the fourth day after inoculation, and tissue suspensions were prepared of the cerebra (with thalamus), cerebella, midbrains together with pons, and medulla. Medulla was examined separately because it showed a resistance to necrosis in the 7-day and older mice during Coxsackie B-3 virus infection. The 7-day-old mice were included in this test to represent the earliest age at which traces of myelin could be observed in any part of the brain.

The virus was recovered from all parts of the brain at all ages (Table V). The titers appeared to decrease with the increasing age of the mouse, but there were no dramatic changes to accompany the abrupt disappearance of the pathological changes, nor were there any marked differences in the titers

TABLE V

Coxsackie B-3 virus recovered from the brain of mouse* on the fourth day after inoculation by the subcutaneous route

Age of mice (days)	$-\text{Log } LD_{50}$ in mice, subcutaneously			
	Cerebrum	Brain stem	Cerebellum	Medulla
4	5.80	5.75	5.75	5.75
7	5.00	4.75	4.80	4.50
17	4.25	2.64	2.75	2.75
70	3.63	2.25	2.00	2.50

*The pooled brain of 15 to 30 mice was examined for each age group.

when the various parts of the brain were compared. However, the cerebrum in the two older age groups yielded a virus titer between 1 and 2 logs higher than the rest of the brain.

The observations from this test confirmed that the suckling mouse was a far more susceptible host than the continuous human kidney cell cultures to the Coxsackie B-3 virus strain used (Table IV). However, both the in vivo and the in vitro tests showed that the titer of the Coxsackie B-3 virus, isolated on the fourth day of infection, had little or no bearing upon the extent of the pathological changes in the mouse brain (Tables III, IV, and V), nor did it vary proportionally to the increase in myelination.

Discussion

The present studies indicate that in experimental Coxsackie B-3 infection of the mouse, the neuropathogenicity is confined entirely to the *suckling* animal. However, it is of interest to find that the brain tissue of weanling and *adult* mice can support the presence of virus for a period of 10 to 13 days after inoculation. Equally interesting and provocative is the fact that the cerebellum is not susceptible at any age to damage by the virus strain used, although it will support the multiplication of virus in suckling mice to the same titer as the highly susceptible cerebral area.

In most other experimental neurotropic infections, writers have observed a rising resistance to brain damage with increasing age if the peripheral route of inoculation was used (8, 9, 11, 12, 14), whereas at the same time the host remained completely susceptible to infection by the intracerebral route. This led to the proposal that certain peripheral barriers must be brought into operation as the animal ages (14). Since Coxsackie group B infection does not produce encephalitis in the older mouse, these peripheral barriers will not complicate any analysis of the changing susceptibility in this animal and it would appear that the virus–host system described in this paper is ideal for the study of the intrinsic barriers of the central nervous system. A start has been made by considering one of these factors — the myelination process.

A comparison of several routes of inoculation under different host-age conditions indicates that there is some sudden change within the brain itself, and furthermore this change takes place at the age of most active myelination, i.e., between 7 and 10 days. Folch (2) observed very striking chemical changes in the composition of the brain of the mouse at this very stage of development. Thus it is tempting to relate the myelination process to the resistance, for, at the one extreme, the most extensive lesions were observed in the brain before the age of 7 days at a time when it was completely free of demonstrable myelin, while, at the other extreme, there was a total absence of histological lesions in the 15-day-old animals in which traces of myelin could be recognized throughout the whole brain. However, when the separate parts of the brain were analyzed, the relationship appeared to be less distinct, and for the cerebellum, the resistance to the Coxsackie B-3 infection appeared to be quite independent of the state of myelination. It is highly probable that the methods employed were not sensitive enough to demonstrate the very early stages of myelination (1). Also, it may be that the resistance to the virus

infection depends not so much upon the physical presence of myelin, as upon the activity of the cells during myelination.

The mapping of the areas of the brain of particular susceptibility to Coxsackie B-3 virus has been undertaken for two reasons; firstly, it seems to provide indispensable information, if one wants to study the intrinsic factors acting in experimental neurotropic virus infections, and secondly, it is felt that such experimental data may contribute to the understanding of idiopathic nervous disorders in the human patient who survives an earlier encephalitis. This is becoming increasingly important as may be seen from the report of Lennette *et al.* (10) that Coxsackie virus encephalitis was commoner than mumps, herpes, or arbor virus encephalitis in California in 1955–57. Furthermore, since non-fatal cases of Coxsackie infection in man greatly exceed the few recorded fatal cases, one wonders what sequelae may result in children infected at an age when resistance is high enough to prevent a fatal outcome. In at least one case, the development of Parkinson's syndrome has been associated with Coxsackie B-2 infection (17) in a woman aged 54 years.

However, the fact that, in the few post-mortem reports of fatal Coxsackie group B encephalomyocarditis in newborn babies, the cerebellum (5, 6, 15, 16) in man appeared to be susceptible underlines the inherent dangers in drawing too close a comparison between the experimental host and man until the basic mechanism of the host–virus relationship is better understood.

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THE OCCURRENCE IN THE HOST PLANT OF PHYSIOLOGICALLY ACTIVE GUMS PRODUCED BY CORYNEBACTERIUM INSIDIOSUM AND CORYNEBACTERIUM SEPEDONICUM[1]

J. F. T. Spencer and P. A. J. Gorin

Abstract

Corynebacterium insidiosum and *Corynebacterium sepedonicum*, the causative agents of bacterial wilt of alfalfa and of potato ring rot, produce viscous polysaccharide solutions when grown in shaken culture. These substances contain fucose, a sugar rarely found in polysaccharides of land plants. Hydrolyzates of polysaccharides extracted from alfalfa and potato plants infected with these organisms also contained fucose, while extracts of healthy plants did not. Alfalfa cuttings placed in dilute polysaccharide solutions wilted rapidly. These findings suggest that these polysaccharides are probably formed in the vascular systems of infected plants and that if such is the case they contribute to the development of the disease in these plants.

Introduction

Polysaccharides, which are often formed during bacterial growth, are known to cause wilting of plants (1). The wilting is caused by plugging of the vascular tissue by the polysaccharides. *Corynebacterium insidiosum* and *C. sepedonicum* form gums during artificial culture, in sufficient concentrations to cause rapid wilting of cuttings from alfalfa seedlings when the latter are placed in the supernatant liquid from cultures of these organisms. The modes of action of these two pathogens, however, have not been definitely determined. Paquin, Lachance, and Coulombe (4) have recently demonstrated, in a study of bacterial ring rot of potato, that *C. sepedonicum* did not produce detectable quantities of pectic enzymes, which are responsible for the manifestation of disease symptoms in host plants infected by *Erwinia* species, *Pythium* de *Baryanum*, and *Botrytis cinerea*, for example. They suggested that mechanical action or a feebly toxic bacterial secretion might be responsible for the disease symptoms. If the gum, produced copiously during artificial culture, was produced during growth in the infected plant, it could serve as the mechanical agent bringing about wilting in alfalfa, and ring rot symptoms in potatoes. In this paper, evidence is given that polysaccharide gums are produced during the growth of these two pathogens in the infected host plants.

Experimental

Authentic cultures of *Corynebacterium insidiosum* ATCC No. 10253 and *C. sepedonicum* ATCC No. 9850 were obtained from the American Type Culture Collection. These cultures were maintained on a medium containing 1.5% glucose, 1.0% yeast extract, 0.5% $CaCO_3$, and 1.3% agar.

[1]Manuscript received October 5, 1960.
Contribution from the Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan. Presented in part at the annual meeting of the Royal Society of Canada, Symposium on Carbohydrate Chemistry, June 6–8, 1960.
Issued as N.R.C. No. 6198.

Fermentations

The organisms were grown in a medium containing 1.5% glucose, 1.0% yeast extract dialyzate, and 0.2–0.5% calcium carbonate. The yeast extract solution was dialyzed before use to remove yeast polysaccharides. For production of small quantities of gum, the medium was dispensed in 100 ml quantities in 500- ml Erlenmeyer flasks, sterilized by autoclaving, and inoculated from a slant of the desired culture. The flasks were incubated at 25° C on a rotary shaker, eccentricity ½ in., operated at 230 r.p.m. For production of larger quantities of gums the organisms were grown in the same medium under agitation in fermentors of the 5-liter glass jar type having a working volume of three liters.[2]

Cells were removed in a Sharples supercentrifuge, or when the culture was very viscous in a Spinco ultracentrifuge. Culture supernatants which contained a good deal of suspended matter after concentration were again centrifuged in the ultracentrifuge. After centrifugation the viscous solutions were evaporated to as small a volume as possible and the polysaccharides precipitated by addition of 5 volumes of acetone. The materials were recovered by filtration and then redissolved in water and the solutions treated with Amberlite IR120 and Dowex 1. After filtration and evaporation to small volumes, the polysaccharides were reprecipitated, washed with ethanol, and dried. On the average, yield of *C. insidiosum* polysaccharide was 2 g/liter and that of *C. sepedonicum* 2.5 g/liter.

Each polysaccharide contained D-glucose, D-galactose, and L-fucose residues as shown by acid hydrolysis followed by cellulose column chromatography of the hydrolyzate. The ratios of these sugars were measured by separating them on Whatman No. 1 filter paper pretreated with ammonium hydroxide (solvent ethyl acetate 10: pyridine 4: water 3 v/v) and estimating the amounts present by the benzidine colorimetric method (3). For the *C. insidiosum* polysaccharide the ratio of galactose: glucose: fucose, was 0.70: 0.46: 1 and for *C. sepedonicum* it was 1.55: 0.93: 1. $[\gamma]_D$'s of the above polysaccharides were $-74°$ (*c*, 0.1 H_2O) and $+87$ (*c*, 0.1 H_2O).

Tests of Wilting Powers of Gums

The effect of solutions of purified polysaccharides on cuttings from mature alfalfa is shown in Fig. 1. Cuttings taken from young plants wilted in 10–15 minutes, much more readily than did those from mature plants. However, slips from mature plants placed in solutions containing 0.002% polysaccharide from *C. insidiosum* wilted after about 10 days. An aliquot of the culture broth was dialyzed against distilled water, to remove any toxic compounds of low molecular weight. No wilting was observed in alfalfa slips placed in dialyzates of culture broths, while slips in the dialyzed material wilted as rapidly as those in the crude broth. It was assumed from this that no free toxins of low molecular weight were produced in artificial culture.

Detection of Fucose in Infected Plants

The plant materials used in this study were kindly supplied by Dr. M. W. Cormack.

[2]Manufactured by New Brunswick Scientific Company, New Brunswick, New Jersey.

PLATE I

FIG. 1. Effect of polysaccharides from *C. insidiosum* on alfalfa cuttings; (1) control, (2) 0.002% polysaccharide, (3) 0.02% polysaccharide, (4) 0.2% polysaccharide.

Five hundred grams of alfalfa roots, infected by *C. insidiosum*, were cut into small fragments and heated 18 hours at 100° C in water (3 liters). The digest was filtered and evaporated to 200 cc and an excess of ethanol (1.5 liters) added. The precipitated polysaccharide-containing material was filtered off, washed with ethanol, and dried (yield 76 g). A portion (500 mg) was hydrolyzed in *N* sulphuric acid (10 cc) for 18 hours at 100°, the solution neutralized ($BaCO_3$), and filtered. The solution was treated with mixed Amberlite IR120 and Dowex 1 to remove salts and then it was filtered and evaporated to a syrup. Paper chromatographic examination in butanol: ethanol: water (40: 11: 19 v/v), ethyl acetate – acetic acid – water (9: 2: 2 v/v) and ethyl acetate – pyridine – water (10: 4: 3 v/v) using *p*-anisidine hydrochloride as spray reagent (2) showed a trace of material cochromatographing with fucose in the presence of a large excess of arabinose. The latter sugar is a constituent of the plant polysaccharides, which are present in much larger quantity than is the bacterial polysaccharide. Polysaccharides isolated by this procedure from healthy plants showed no trace of fucose. Similar techniques were used for detection of fucose in potatoes infected with ring rot.

Discussion

The occurrence of fucose was taken as strong presumptive evidence that gums produced by the pathogens were present in the plants. This sugar is a major component of the gums produced by these two organisms, but is not commonly found in polysaccharides of land plants. Analysis of hydrolyzates of polysaccharides from the host plants showed that fucose was present in infected plants and was not detectable in healthy controls. These results do not exclude the possibility that further toxic materials may be formed in vivo by the organisms. However, dialyzates of culture supernatants do not cause wilting, the polysaccharides used in these studies were treated with mixed bed ion exchange resins to remove any acidic or basic toxins which may have been present, and Paquin *et al.* (4) have shown that pectinases are not responsible for the development of bacterial ring rot of potato. Since the gums formed by these organisms cause wilting of plants, and since it has been shown that they are probably formed by the pathogens in the infected hosts, these gums are very likely responsible for some, at least, of the disease symptoms in alfalfa. The effect of polysaccharide solutions on potato cuttings was not determined, but in view of the effect of high molecular weight compounds on plants demonstrated by Hodgson *et al.* (1), the effect of *C. sepedonicum* polysaccharide on the infected plant is probably deleterious.

Acknowledgments

The authors wish to express their thanks to Dr. M. W. Cormack, Canada Agricultural Research Laboratories, for advice and for supplying infected plants of alfalfa and potato; Mr. J. A. Spencer, Magrath, Alberta, for suggestions which led to the study of the problem; and Mr. N. R. Gardner and Miss Sheila Lubin for technical assistance.

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ACTIVE COMPONENTS OF ANTIGLOBULIN SERA REACTING WITH RED CELLS SENSITIZED WITH NATURAL AND IMMUNE ANTI-A HAEMAGGLUTININS[1]

ROMA Z. HAWIRKO AND F. C. C. WONG

Abstract

Four antihuman globulin sera (AHG) were prepared by immunizing rabbits with (1) purified gamma globulin and (2) anti-A haemagglutinins purified by elution from sensitized cells namely, (*a*) natural anti-A from a male who had received no known antigenic stimulus, (*b*) immune anti-A+B after delivery, and (*c*) immune anti-A from the cord blood of an infant with mild ABO haemolytic disease. The highest dilution of AHG sera agglutinating haemagglutinin-sensitized cells was essentially the same with the natural (2*a*) and immune (2*b*) sera with titers from 1:40 to 1:320 while with the immune (2*c*) serum the titers ranged from 0 to 1:20. The antihuman gamma globulin (AHGG) serum did not agglutinate cells sensitized with the natural anti-A and showed a titer of 1:2560 with cells sensitized with immune anti-A+B.

Micro-Kjeldahl analysis of the four AHG sera precipitated with gamma globulin showed marked differences in μg nitrogen per ml of gamma globulin and of antigamma globulin and in the ratio of antibody to antigen at maximum precipitation. There appeared to be a quantitative relationship between the activity of serum in the antiglobulin test and the ability of serum to combine specifically with gamma globulin. Quantitative analysis showed that the amount of gamma globulin in μg nitrogen required for maximum precipitation of the natural AHG serum was the same as required for neutralization of the serum agglutinating natural anti-A-sensitized cells but only one-third was required for serum agglutinating cord anti-A-sensitized cells. The gamma globulin (1) serum, which was inactive with cells sensitized with natural anti-A, restored the agglutinating activity of the gamma-globulin-neutralized natural AHG (2*a*) serum.

Electrophoretic studies showed that normal rabbit serum, natural AHG rabbit serum, and gamma globulin AHGG contained the same globulin components except for the alpha one fraction. Both the AHG sera were characterized by a new alpha component while the gamma globulin AHG showed an additional new alpha component. The significance of these findings is discussed.

Introduction

Isoimmunization, which results in haemolytic disease of the newborn, has been shown independently by Race and by Wiener (8, 9) to be characterized by appearance of a non-saline-agglutinating or incomplete antibody. The antiglobulin test, introduced by Coombs, Mourant, and Race in 1945 (2), has been used extensively for the study of incomplete Rh and other antibodies including the anti-A haemagglutinin. The antihuman globulin sera (AHGG) prepared in rabbits immunized with whole human serum, agglutinated red blood cells previously sensitized with incomplete haemagglutinin. The active component in the AHG rabbit serum is reported to be an antigamma globulin (3) which reacts specifically with the globulin part of the incomplete Rh antibody attached to the surface of the red cells. This finding is supported by the neutralization of the agglutination by the addition of purified gamma globulin (3).

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Contribution from the Department of Microbiology, University of Manitoba, Winnipeg, Manitoba.

Since different antiglobulin sera showed wide variations of activity with non-saline agglutinating antibody, it is proposed that quantitative differences may be due to the concentration of the active antigamma globulin fractions while qualitative differences would suggest that the active components are made up in part of other than antigamma globulin. In the ABO blood group system, the haemagglutinins are reduced to zero or almost zero in agamma-globulinaemia, but in plasma fractionation they separate out in the non-gamma globulin component. The present study was undertaken to determine the active components of the antiglobulin sera prepared by immunizing rabbits with purified gamma globulin and with purified incomplete anti-A or anti-A+B from immune and non-immune donors. The antiglobulin sera were tested: first, for agglutination of red cells sensitized with natural or with immune anti-A or anti-A+B; second, for the effect on the addition of gamma globulin on the agglutination; third, for the nitrogen content of precipitates with gamma globulin; and finally for globulin components by paper electrophoresis.

Material and Methods

Two groups of haemagglutinins were used: first, natural anti-A from group B male donors (Halvorson, Wong, and Turbovsky) who had received no known antigenic stimulus; and second, immune anti-A+B from a group O donor after delivery of a group A infant (Penner); and immune anti-A from a cord blood serum of a group A baby with subclinical ABO haemolytic disease (Boadway).

Purified incomplete haemagglutinins were obtained by elution from A_2 cells, which were sensitized with anti-A or anti-A+B at 37° C for 2 hours, washed six times in ice-cold saline, and suspended in a volume of saline. The antibodies were eluted from the surface of the cells into saline by heating in a 56° C water bath for 5 minutes with gentle agitation. Although the purified haemagglutinins from the immune donors did not agglutinate the red blood cells except on the addition of the antiglobulin sera, the haemagglutinins from the natural donors showed a saline titer of 1:64.

Human gamma globulin kindly supplied by Dr. Bruce Chown, when examined by paper electrophoresis (1) using a barbitone buffer pH 8.6 at 5 ma for 16 hours, was found to be homogeneous and electrophoretically identical with the gamma globulin component of normal serum. The eluted anti-A from the natural (Halvorson) serum was also examined and found to show the same electrophoretic mobility as gamma globulin.

Antiglobulin sera (AHG) were prepared by immunizing four groups of rabbits as follows: the first group with human gamma globulin; the second group with natural anti-A (Halvorson); the third group with immune anti-A+B (Penner); and the fourth group with immune anti-A from cord blood (Boadway). The method of immunization was that of Kabat (4) using two rabbits for each group except the fourth group, in which only one rabbit was immunized because of the limited amount of cord serum. Prior to testing, the saline and non-specific agglutinins were removed from the serum by heating for 1 hour at 70° C and by an absorbing with washed unsensitized group O and A cells. The sera to be examined electrophoretically were absorbed but not heated.

The activity of the antiglobulin sera on each of the haemagglutinins was

measured by the antiglobulin test (7). A_1 cells were sensitized with serial dilutions of eluted haemagglutinins ranging from 1:4 to 1:256 at 37° C for 2 hours. The cells were then washed six times in saline and aliquots from each were tested for agglutination with the AHG sera added in serial dilutions ranging from 1:4 to 1:5120. In repeated tests the haemagglutinins from the natural donors showed no evidence of agglutination in the saline controls, which, as noted above, previously showed a saline titer of 1:64. This apparent anomaly was resolved when it was shown that the saline agglutinins were removed during the antiglobulin test in the washing of the sensitized cells at room temperature, after the second washing the titer had decreased to 1:8 and after the third washing to zero.

The effect of gamma globulin on the agglutinating activity of the antiglobulin serum, produced by the injection of natural anti-A, was tested on cells sensitized with haemagglutinins from the natural donors (Halvorson, Wong, and Turbovsky) and from the immune donor (Boadway). Decreasing amounts of gamma globulin from 100 to 1.56 μg were added to 0.2 ml of antiglobulin serum and allowed to react overnight. The so-treated serum, in serial dilution, was then used to test the agglutination of red cells that had been sensitized with each of the haemagglutinins diluted 1:20. One test was carried out by adding the AHGG produced by the injection of gamma globulin to the gamma-globulin-treated natural AHG and then testing for the agglutination of sensitized cells.

The antigamma globulin precipitin content of the four antiglobulin sera was determined by the Markham micro-Kjeldahl method (6). Appropriate amounts of gamma globulin, so as to insure antibody excess, were added to dilutions of the sera in duplicate. The precipitates were allowed to form at 37° C for 1 hour and at 10° C for 7 days and then washed well in ice-cold saline. From the total nitrogen content of each specific precipitate, of serum controls, and of gamma globulin, the amount of antigamma globulin nitrogen and the ratio of antibody to antigen nitrogen in the equivalence zone was calculated for each of the antiglobulin sera.

The gamma globulin AHGG and the natural AHG sera were examined electrophoretically. The peaks of mobility, estimated with a densitometer, were compared with normal rabbit serum and with natural AHG after absorption with cells sensitized with anti-A.

Results

The sensitizing activity of the anti-A haemagglutinins, expressed as the highest dilution required for the sensitization of A_1 cells to agglutinate with the antiglobulin sera is recorded in Table I (*a*). The titers of the natural anti-A haemagglutinins were 1:256 with the natural AHG serum, 1:32 and 1:16 with the immune AHG sera, and surprisingly did not react at all with the gamma globulin AHG serum. The titers of the haemagglutinins from the two immune donors were essentially the same with each of the antiglobulin sera, ranging from 1:16 to 1:32. On the basis of these findings a haemagglutinin dilution of 1:20 was selected for the sensitization of cells in subsequent tests, in order to eliminate the laborious cross titrations and to conserve the sera.

The agglutinating activity of the AHG sera, expressed as the highest dilution

required for the agglutination of A_1 cells sensitized with a 1:20 dilution of haemagglutinin, is recorded in Table I (*b*). The dilutions of AHG sera agglutinating cells sensitized with natural haemagglutinin ranged from 0–1:320, with immune (Penner) from 0–1:2560 and with immune (Boadway) from 1:40–1:80. The titers of the natural and of the immune (Penner) AHG sera were essentially the same, while the immune (Boadway) was, by comparison, relatively inactive. The gamma globulin AHGG, as noted above, failed to agglutinate cells sensitized with the natural haemagglutinin and also differed from the other sera by agglutinating to a titer of 1:2560 cells sensitized with the immune (Penner) haemagglutinin.

TABLE I

Cross titration of antiglobulin sera anti-A-sensitized cells

	Rabbit antisera (AHG)				
Haemagglutinin-sensitized cells	No. 1 Gamma glob.	No. 2 Natural anti-A	No. 3 Immune anti-A+B (Penner)	No. 4 Immune anti-A (Boadway)	Saline
	(*a*) Titration of haemagglutinins				
Natural anti-A	0	1:256*	1:32	1:16	0
Immune anti-A+B (Penner)	1:32	1:32	1:32	1:64	0
Immune anti-A	1:16	1:16	1:64	—	0
	(*b*) Titration of antiglobulin sera				
Natural anti-A	0	1:320†	1:320	1:20	0
Immune anti-A+B	1:2560	1:320	1:320	0	0
Immune anti-A	1:40	1:40	1:80	—	0

*Highest dilution of haemagglutinin required for the sensitization of cells for agglutination with antiglobulin sera.
†Highest dilution of antiglobulin sera agglutinating cells sensitized with 1:20 haemagglutinin; 0 refers to absence of agglutination; — omitted.

As expected, the gamma globulin neutralized the ability of the natural AHG serum to agglutinate cells sensitized either with the natural or with the immune (Boadway) haemagglutinins (Table II). The amount of gamma globulin,

TABLE II

Effect of gamma globulin on natural anti-A antiglobulin serum

	Rabbit antisera (AHG)			
Haemagglutinin-sensitized cells	No. 1	No. 2	No. 2 + G.G.*	No. 2 + G.G. + No. 1
	Natural anti-A			
Halvorson	0	1:320†	0	1:160
Wong	0	1:160	0	1:160
Turbovsky	0	1:320	0	1:320
	Immune anti-A			
Boadway	1:32	1:32	0	—

*Purified solution of human gamma globulin.
†Highest dilution of antiglobulin serum which agglutinates sensitized cells.

expressed as μg nitrogen per ml required for the neutralization of the natural AHG serum, was 0.478 for cells sensitized with natural anti-A whereas 0.159 was sufficient for cells sensitized with immune cord anti-A. When the gamma globulin AHGG serum was added to the neutralized natural AHG serum, the resulting mixture again agglutinated the red cells sensitized with natural anti-A, although the gamma globulin AHGG was unable to bring about the agglutination in the standard antiglobulin test.

The antibody nitrogen content of the four antiglobulin sera precipitated with human gamma globulin showed marked variation (Table III). The antibody

TABLE III

Nitrogen precipitated by gamma globulin added to AHG sera

Gamma globulin, μg N/ml	Total, μg N/ml	Antibody, μg N/ml	Ratio antibody: γ globulin
No. 1			
0.511	1.400		
0.613	1.428		
0.715	1.477		
0.916	1.666		
0.919*	2.486*	1.567	1.705
1.020	2.324		
1.226	1.023		
1.328	0.280		
No. 2			
0.081	0.250		
0.102	0.501		
0.204	1.180		
0.214	1.205		
0.316	1.390		
0.484*	1.680*	1.196	2.471
0.511	1.420		
0.560	1.385		
0.613	1.225		
0.818	0.910		
No. 3			
0.408	0.820		
0.448	0.860		
0.511	0.975		
0.613*	1.236*	0.623	1.016
0.672	1.220		
0.715	1.036		
0.728	1.034		
0.784	0.925		
0.809	0.875		
0.817	0.856		
1.025	0.700		
No. 4			
0.020	0.280		
0.041	0.420		
0.051	1.008		
0.051*	1.036*	0.985	19.313
0.061	0.896		
0.071	0.700		
0.102	0.280		

*Equivalence zone.

antigen ratio, which indicates the combining power of the antigamma globulin, at maximum precipitation was highest in the immune (Boadway) AHG, followed by natural AHG, the gamma globulin AHGG, and the immune (Penner) AHG with values of 19.313, 2.471, 1.705, and 1.016 respectively. The data are presented graphically in Fig. 1. In general, there was no apparent relationship between the antibody antigen ratio and the amount of serum required to bring about agglutination in the antiglobulin test, with the exception of the immune (Boadway) AHG serum, where the highest antibody antigen ratio was observed and the greatest amount of serum was required to bring about agglutination. The amount of gamma globulin required to produce maximum precipitation with 1 ml of natural AHG serum was 0.484 μg nitrogen per ml.

Electrophoretic studies showed that the gamma globulin AHGG, the

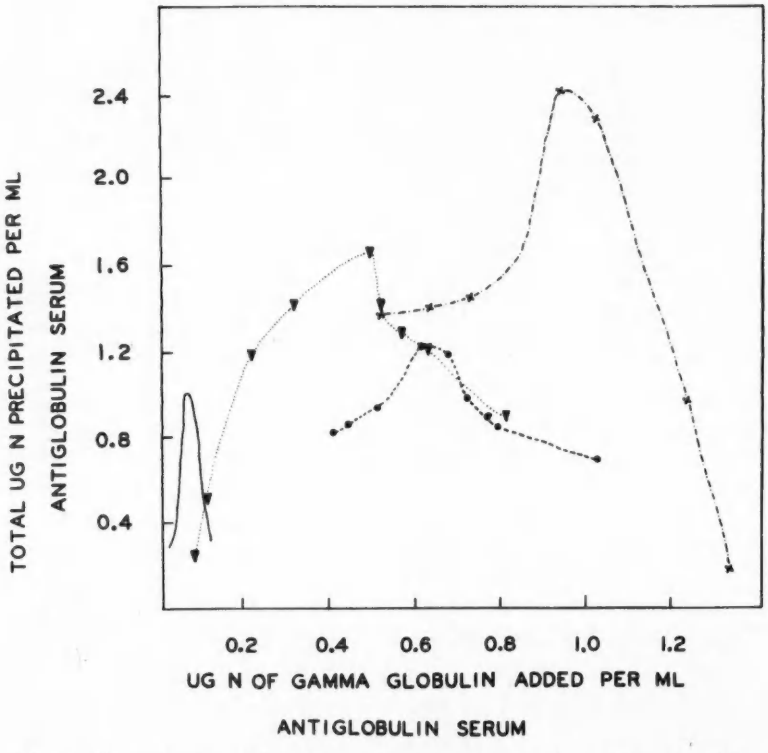

FIG. 1. Microestimation of the nitrogen content of the precipitates formed by all the prepared antiglobulin sera with varying amounts of gamma globulin. Antiglobulin sera: (1) gamma globulin ×, (2) natural anti-A ▼, (3) immune anti-A •, (4) immune cord anti-A ———.

natural AHG, and normal rabbit serum contained the same globulin except for one component. As indicated in Fig. 2, both antiglobulin sera were characterized by a new alpha fraction while the gamma globulin AHGG showed an additional new alpha fraction. The new alpha fraction was removed from the natural AHG serum by absorption with cells sensitized with natural anti-A haemagglutinin.

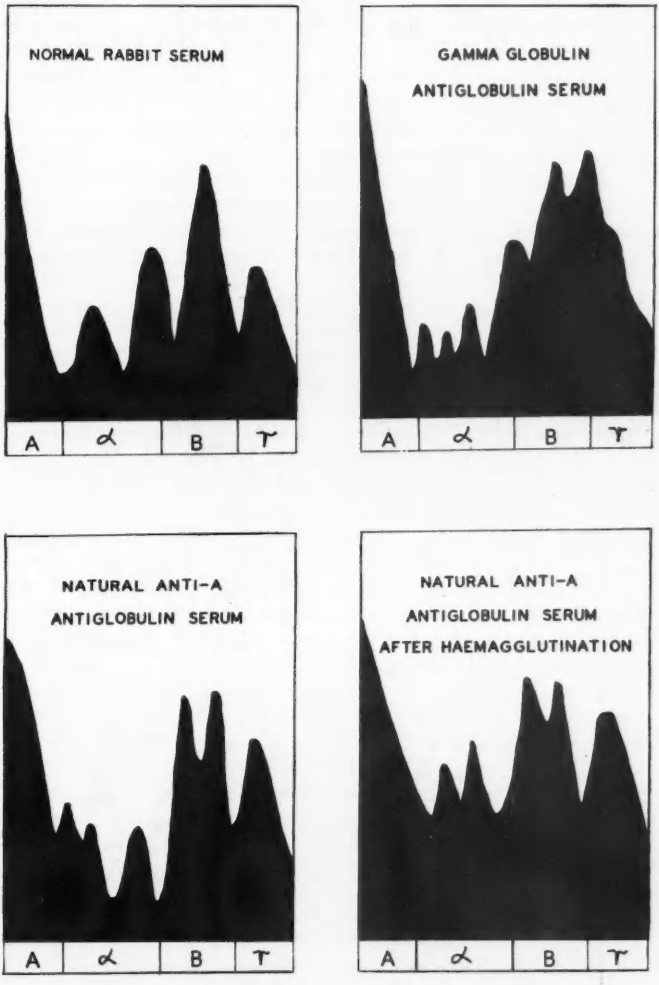

FIG. 2. Electrophoretic patterns of antiglobulin sera.

Discussion

A relationship between the amount of AHG sera required to agglutinate sensitized cells and the antibody antigen ratio as determined by precipitation with gamma globulin was not readily apparent. The indirect test, which showed that the amount of gamma globulin required to neutralize the ability of the natural AHG serum to agglutinate cells sensitized with natural haemagglutinins was the same as the amount precipitated by the AHG serum (0.478 μg nitrogen per ml compared to 0.484), gave evidence of a quantitative relationship between the activity of the serum in the antiglobulin test and the ability to combine specifically with gamma globulin.

The finding that the amount of AHG serum required to agglutinate sensitized cells varied according to the type of sensitizing haemagglutinin was confirmed quantitatively by the indirect test, which showed that the amount of gamma globulin required to neutralize the ability of natural AHG serum to agglutinate cells sensitized with immune cord haemagglutinin was only 0.157 μg nitrogen per ml, while inhibition of agglutination by the same AHG serum of cells sensitized with natural haemagglutinin required 0.478. Since the amount of gamma globulin required to neutralize the agglutination of cells sensitized

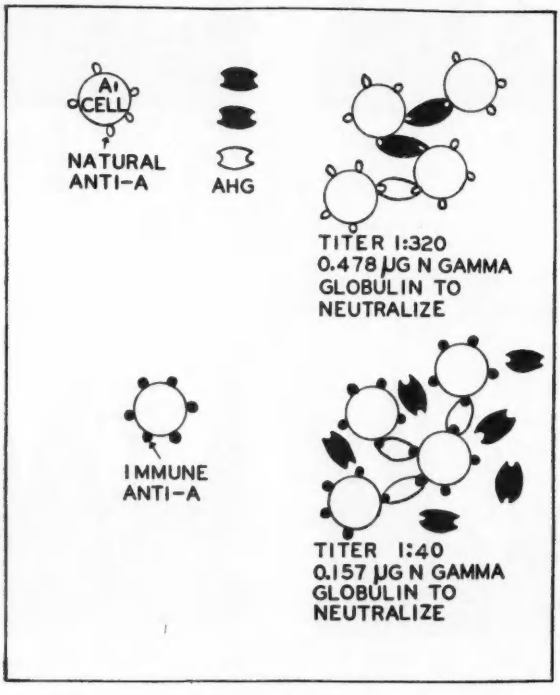

FIG. 3. Schematic illustration of antiglobulin test.

with immune cord anti-A was approximately one-third of the total precipitated by AHG serum, we suggest that only some of the antigamma-globulin molecules were utilized. Conversely, the amount required to neutralize the agglutination of cells sensitized with natural anti-A was the same as the total precipitated by the AHG serum, and therefore all of the antigamma-globulin molecules took part in the agglutination. This concept, expressed graphically in Fig. 3, would also explain the varying amounts of AHG serum required to agglutinate the cells sensitized with the two types of anti-A on the basis of the number of active molecules present per unit volume of serum, that is, the greater the number of active molecules, the less serum required for agglutination.

The gamma globulin AHGG serum differed from the other three in two respects. It failed to agglutinate cells sensitized with natural anti-A and its titer of 1:2560 compared to 1:320 with cells sensitized with immune anti-A. If it can be inferred that the gamma globulin used for the neutralization and for the production of antiglobulin serum is immunologically heterogeneous with more than one determinant group, it would induce the production of more than one antibody each specific to one of the determinant groups present. Similarly, if the natural anti-A, which had been specifically eluted from the sensitized red blood cells, is immunologically homogeneous with only one determinant group, it would induce only one antibody. The combined action of the antibodies to the two determinant groups present in the gamma globulin AHG serum might then account for the high titers with cells sensitized with immune anti-A+B while with cells sensitized with natural anti-A it is suggested that one of the determinant groups exerts a blocking effect and prevents agglutination. This may explain the ability of the gamma globulin AHG serum to reactivate the gamma-globulin-neutralized natural AHG serum. If the gamma globulin possesses two active determinant groups, one may combine with the second alpha globulin fraction of the gamma globulin AHG leaving the other determinant group free to restore the agglutinating activity.

The new alpha fraction, which appeared in natural AHG serum following immunization, was removed by absorption with cells sensitized with natural anti-A haemagglutinin (Fig. 2) and seems to be the active component of the AHG serum. On the basis of the neutralization of the serum with gamma globulin, correlated with the precipitin test, it would appear that the active component is entirely an antigamma-globulin fraction.

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STUDIES ON THE IRRADIATION OF MICROORGANISMS IN RELATION TO FOOD PRESERVATION

I. THE COMPARATIVE SENSITIVITIES OF SPECIFIC BACTERIA OF PUBLIC HEALTH SIGNIFICANCE[1]

I. E. ERDMAN,[2] F. S. THATCHER,[2] K. F. MACQUEEN[3]

Abstract

The limits of tolerance to gamma irradiation have been determined for 21 cultures representative of the following bacterial species of public health significance in foods: *Staphylococcus aureus, Escherichia coli* (types I and II), *Aerobacter aerogenes, A. cloacae, Streptococcus faecalis, Salmonella gallinarum, S. tennessee, Mycobacterium tuberculosis, Clostridium botulinum* (types A and E). A several-fold difference in radiation sensitivity has been demonstrated, specific sensitivity being influenced by the nature of the suspending medium during irradiation.

Spores of *C. botulinum*, type A, were more resistant than those of type E, while six strains of staphylococci of differing phage pattern showed closely similar tolerances. The most sensitive species among those tested was *M. tuberculosis*. The coliform organisms were more sensitive than the salmonellae, which, in turn, were more sensitive than the staphylococci and *S. faecalis*. Destruction of coliform bacteria in irradiated foods cannot, therefore, be used as an index of adequate pasteurization.

Staphylococcal emetic toxin present in pork sausage and suspended in culture filtrates was destroyed by irradiation at comparatively low levels in contradistinction to the findings for the toxin of *C. botulinum*.

Introduction

The preservation of foods by irradiation, including the application of gamma rays from a cobalt^{60} source, has been the subject of much research (2, 4, 6, 7, 8, 11, 12, 13, 15). Several studies emphasize the commercial potentialities of the method by the use of radiation intensities which either accomplish sterilization or which reduce the numbers of contaminant organisms and hence bring about a prolongation of "shelf-life" of the treated food (2, 7, 12, 13). Treatments of the latter kind are referred to as "pasteurizing doses" of irradiation.

This paper reports investigation of the comparative resistance to gamma irradiation of a number of bacteria and of specific toxins commonly of public health significance in foods, and of the influence of different suspending media during irradiation upon specific resistance.

Materials and Methods

Cultures used in these studies included (*a*) six coagulase-positive strains of *Staphylococcus aureus* of different phage patterns: one strain isolated from pastry involved in a food-poisoning episode, three of clinical involvement, and two isolated from cheese; (*b*) the coliforms *Escherichia coli* type I, *E. coli*

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[2]Department of National Health and Welfare.
[3]Atomic Energy of Canada Limited.

type II, *Aerobacter aerogenes* type I, and *Aerobacter cloacae*, all of which were isolated from foods; (*c*) *Streptococcus faecalis* isolated from a food plant; (*d*) two species of *Salmonella* (*S. gallinarum* and *S. tennessee*); (*e*) a B.C.G. strain of *Mycobacterium tuberculosis*; (*f*) *Clostridium botulinum*: one type A strain and five type E strains.

The strains of staphylococci, the coliforms, the salmonellae, and the enterococcus were grown in nutrient broth (Difco) containing 0.3% yeast extract (Difco). Cultures incubated at 37° C for 24 hours were used for irradiation directly or after addition to chopped beef, or were centrifuged and the cells washed and made up to original volume with phosphate dilution buffer at pH = 7.2 (1) for irradiation in buffer suspension. The BCG strain was grown in Dubos medium, separated by centrifugation, and resuspended in the nutrient yeast extract broth prior to irradiation. Stock spore suspensions of *C. botulinum* were prepared by the method of Reed *et al.* (14). With strains producing small spore crops, the techniques of Lund *et al.* (10) were used to increase the yield.

After irradiation of samples, survivors were enumerated by following as closely as possible standard plate count procedures (1). The recovery medium used for most cultures was TGE agar (Difco) containing 0.3% yeast extract. *M. tuberculosis* was counted using surface inoculation of Dubos oleic acid agar, incubation being 4 weeks at 37° C. Spores of *C. botulinum* were counted using a five-tube most-probable-number (MPN) procedure with yeast extract starch bicarbonate broth and by plate count using the yeast extract starch bicarbonate agar of Wynne *et al.* (17). All analyses for clostridia were carried out under an atmosphere of hydrogen in McIntosh Fildes jars. Toxin production in culture fluids was determined using the medium and methods of Duff *et al.* (5).

Meat suspensions of test organisms were prepared by adding spore suspensions or 24-hour broth cultures to comminuted lean beef prepared and inoculated in a sterile cabinet (Fisher Isolator/Lab). For irradiation the broth suspensions were dispensed in 11-ml volumes (to screw-capped vials) chilled to 5° C and held at that temperature except for the irradiation interval, during which samples were in air at room temperature.

Procedures for the enumeration of surviving organisms were begun immediately after completion of irradiation. The density of inocula ranged from 1×10^6 to 1×10^8 per ml or per gram. Aliquots of large test samples were placed in the exposure cell (capacity 24 vials or eight jars) and individually withdrawn at appropriate exposure intervals.

The irradiation equipment was designed and built by Commercial Products Division, Atomic Energy of Canada Ltd., and contained cobalt^{60} with an activity of 4060 curies, the "source" rods being arranged in a "squirrel-cage" container and producing a dose rate of 326,000 r.e.p. per hour, with a uniformity of $\pm 2\frac{1}{2}\%$ throughout the 24-sample positions. Dosimetry was ascertained by the ferrous–ferric sulphate method using a *G* value of 15.5 (16). An initial hourly dose rate was established employing vials similar to those used for subsequent irradiations. The delivered dose was calculated from the established dose rate and exposure time with corrections made for source decay.

Since survival curves are generally exponential (9), plots of percentage survival (log scale) versus radiation dose (arithmetic scale) have been chosen as the most convenient method of presenting results. With this presentation it must be remembered that even when the percentage survival is small there may still be an appreciable number of viable organisms present if the initial numbers are high.

Results

The sensitivities to gamma irradiation of specific non-spore-forming cultures in broth suspension are shown in Fig. 1. Results given are averages of at least two determinations, and where a number of strains were checked, are average values of all determinations. *Streptococcus faecalis* was more resistant than the staphylococci, salmonellae, coliforms, and *M. tuberculosis*, these being listed in descending order of resistance. The low resistance of the last-mentioned species is in marked contrast to its heat resistance as compared to that of the same group of bacteria.

The data expressed in Fig. 2 show the comparative resistance of representatives of types A and E of *C. botulinum*, each of which is more resistant than the non-sporing organisms depicted in Fig. 1. The data obtained by an MPN

TABLE I

The comparative sensitivity to irradiation of specific bacterial cultures suspended in a culture medium and in chopped beef

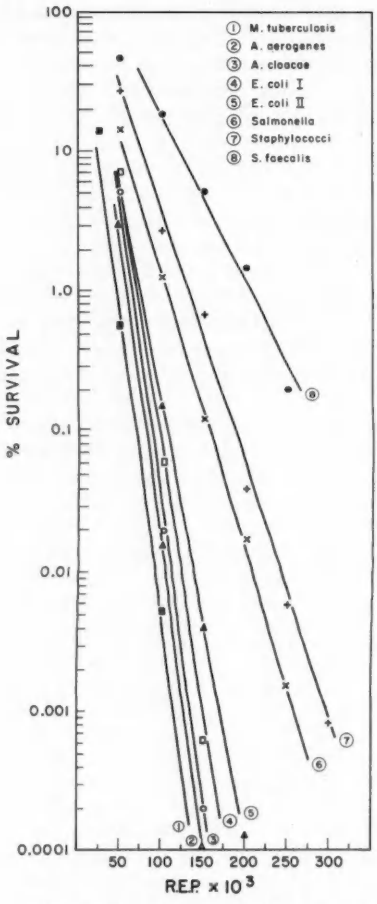
Irradiation dose, r.e.p.×10³	Percentage recovery of cultures irradiated in broth and in meat					
	E. coli I		*Staphylococcus* Cs 68		*Streptococcus faecalis*	
	Broth	Meat	Broth	Meat	Broth	Meat
50	7.2	9.1	—	—	—	—
100	0.060	0.015	7.16	13.10	19.0	9.3
150	0.00063	0.0047	2.32	2.20	5.50	2.73
200	—	—	0.11	0.27	1.50	0.30

TABLE II

The comparative sensitivity to irradiation of *Clostridium botulinum* type A spores suspended in buffer, broth, and meat

Irradiation dose, r.e.p.×10⁶	Percentage survival in:		
	Buffer*	Broth†	Meat‡
0	100	100	100
0.5	7.08	66.91	100
1.0	0.225	3.83	26.09
1.5	0.0005	0.067	0.58
2.0	0	0.00038	0.077

*APHA phosphate buffer at pH =7.2.
†Nutrient broth (Difco) with 0.3% yeast extract.
‡Chopped lean beef.



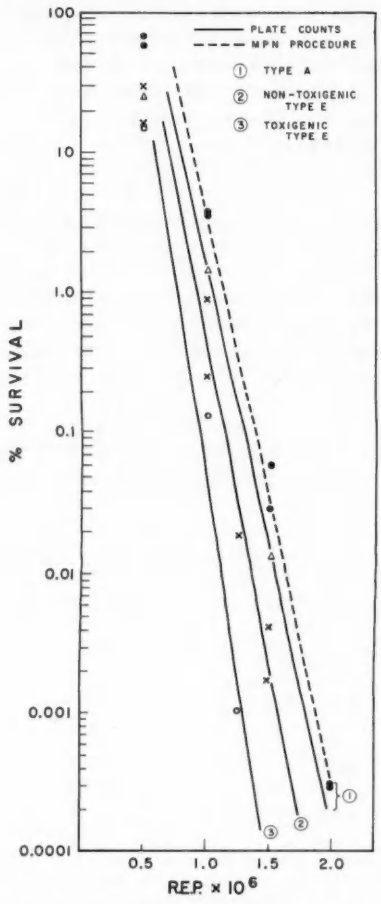


FIG. 1. Comparative irradiation sensitivity of non-spore-forming bacteria suspended in broth culture.

FIG. 2. Comparative irradiation sensitivities of *Clostridium botulinum* spores suspended in broth.

procedure and by plate count provide closely parallel curves. The plate-count method, being technically preferable, was used during the balance of these studies. Spores of type E were less resistant to irradiation than those of type A. Although only data for the "VH" strain[3] of type E are presented, other strains gave similar results. Of all type E cultures tested, the spores of non-toxigenic variants proved to be more resistant to irradiation than those of the toxigenic variants.

[3]Strain designation is Dr. Dolman's.

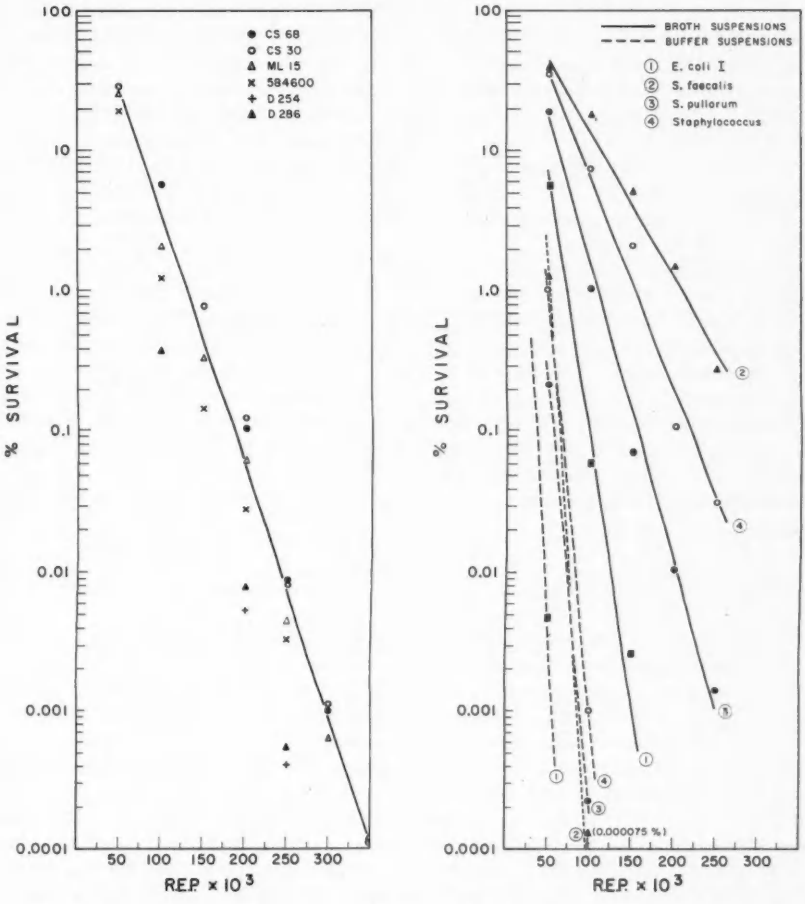


FIG. 3. Variations in irradiation sensitivities of six strains of *Staphylococcus aureus* irradiated in broth suspension.

FIG. 4. Comparative sensitivities of cultures suspended in broth and in buffer during irradiation. (*S. pullorum* is listed as *S. gallinarum* in the 7th edition of Bergey's manual.)

The data in Fig. 3 show the close similarity of irradiation sensitivity among six strains of coagulase-positive *Staphylococcus aureus* of different phage patterns.

Data illustrating the comparative sensitivities of selected strains when suspended in broth and in buffer solution are shown in Fig. 4. All cultures were substantially more sensitive in buffer than in broth. The survival following irradiation at 150,000 r.e.p. in broth for *E. coli*, *S. gallinarum*, *Staphylococcus aureus*, and *Streptococcus faecalis* was 0.001, 0.13, 1.1, and 4.0%, respectively,

while in the buffer after irradiation at 50,000 r.e.p., survival values for the same species were 0.0048, 0.22, 1.0, and 1.25%, respectively. In the buffer suspensions, no survivors were noted in 2-ml portions of suspensions of any of the test cultures following irradiation at 150,000 r.e.p. The order of sensitivity of the respective cultures also differed in the two media. The data in Table I show that the test strains of *E. coli* and *Staphylococcus* were more sensitive when irradiated in broth than in meat, but the reverse was true for *S. faecalis*. The difference in sensitivity conferred by these media ranged from less than 2- to 10-fold. The corresponding difference between broth and buffer exceed 1000-fold at 100,000 r.e.p.

The data presented in Table II show that spore suspensions of *C. botulinum* type A when suspended, respectively, in broth, buffer, or meat expressed differences in irradiation sensitivity with each of the suspending media.

In preliminary trials with crude type A botulinum toxin present in both pork sausage and broth, no effect on toxicity was noted at exposures up to 2.2 million r.e.p. With crude staphylococcal toxin produced in Dolman's medium (3) emetic activity for cats was reduced in potency after irradiation at 110,000 r.e.p. and was absent after 2.2 million r.e.p. The titer of crude staphylococcal toxin with an initial alpha-lysin value of 1:8192 was reduced to 1:1024 following irradiation at 110,000 r.e.p. and to 1:256 following irradiation at 2.2 million r.e.p.

Discussion

The data presented are in agreement with reported wide variations in radiation sensitivity of differing bacterial species (4, 12, 13, 15).

The nature of the suspending medium can influence greatly the relative radiation sensitivity of a specific culture. This suggests that the use of different strains as well as of non-standardized suspending media by different investigators may, in part, explain the reported differences in radiation sensitivities of specific organisms. The effect of the suspending medium on radiation sensitivity may become important in determining effective irradiation treatments for food processing. Chemicals and spices added to foods have been shown to alter bacterial sensitivity to irradiation (15). This suggests the need in commercial application for the determination of specific irradiation doses for each food mixture, particularly if non-sterilizing doses are to be used for foods where the margin between effective bacteriological control and the development of undesirable flavor changes is narrow.

It should be noted, also, that the destruction of coliforms by an irradiation treatment of foods cannot be used as an indication of effective pasteurization as is sometimes inferred in the appraisal of foods pasteurized by heat. This applies because the pathogens *Salmonella* and *Staphylococcus* are substantially more resistant to irradiation than the coliform organisms.

Acknowledgments

The authors wish to thank Dr. B. B. Diena for providing the culture of *M. tuberculosis* used and for his assistance in determining its survival following irradiation, and Dr. C. E. Dolman for providing strains of type E *Clostridium botulinum*.

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STUDIES ON THE IRRADIATION OF MICROORGANISMS IN RELATION TO FOOD PRESERVATION

II. IRRADIATION RESISTANT MUTANTS[1]

I. E. ERDMAN,[2] F. S. THATCHER,[2] K. F. MACQUEEN[3]

Abstract

Using a Co^{60} source, repeated irradiation of survivors of bacterial cultures at a level initially destroying a high proportion of cells gave rise to strains which were more resistant to irradiation than the original cultures. Development of resistance was shown in single strains of *Escherichia coli*, *Streptococcus faecalis*, and *Clostridium botulinum* type A, and in one of three strains of *Staphylococcus aureus*, but not in two strains of *C. botulinum* type E nor in *Salmonella gallinarum*. Changes were noted in phage patterns of the staphylococci, biochemical characteristics of *E. coli*, and toxin production of *C. botulinum* in response to repeated irradiation.

Irradiation of parent and resistant cultures at −78° C did not change their comparative resistance, though each was afforded some protection at this temperature. These findings suggest that the induced resistance is an expression of resistance to the primary effect of irradiation and not to possible toxic substances formed by free-radical interaction.

Introduction

The ability of microorganisms to adapt themselves to environmental conditions which can be demonstrated to be fatal to parent strains is well known. Much work has been done in this field with chemicals and antibiotics, but to date little information is available on the development of radiation-resistant strains. Just as heat-resistant organisms become a problem to food processors and antibiotic-resistant organisms cause difficulties in the treatment of infections, development of radiation-resistant strains may play an important part in determining the effectiveness of radiation processing of foods.

Witkin (11), using ultraviolet irradiation, isolated a culture of radiation resistant *Escherichia coli* strain B which has subsequently been referred to as strain B/r. Radiation was considered to act as a selective agent for a mutation normally occurring at the rate of 10^{-5} per organism per generation. In subsequent experiments Witkin (12) showed that irradiation caused an increase in the spontaneous frequency of the resistant mutants. This means that the irradiation was acting not only as the selective agent but also as the inducing agent for these mutations. Using beta radiation, Koh, Morehouse, and Chandler (7) were unable to show increased resistance in survivors of cultures of *E. coli* after two irradiations. Luckiesh and Knowles (8) demonstrated a doubling in resistance to ultraviolet irradiation by those cells of *E. coli* surviving the first irradiation but no additional increase in resistance after five subsequent irradiations. Their resistant cultures had less metallic sheen on EMB agar,

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Contribution from the Laboratories of the Food and Drug Directorate, Department of National Health and Welfare, Ottawa, and Commercial Products Division, Atomic Energy of Canada Limited, Ottawa.
[2]Department of National Health and Welfare.
[3]Atomic Energy of Canada Limited.

but of nine biochemical reactions tested, the only other change noted was the failure of the resistant strain to ferment dulcitol. Gaden and Henley (4), using gamma irradiation, showed an increased susceptibility to radiation damage when *E. coli* strain 15 was given repeated irradiation exposures, but reported an increased resistance by *E. coli* B and B/r under similar treatment. The increase in resistance diminished with continued subculture and irradiation so that after 20 such transfers changes became so small that it was difficult to tell whether or not a limit had been reached. Pepper, Buffa, and Chandler (9), using cathode ray irradiation, report that spores produced by resistant isolates of one irradiation had survivor patterns similar to spores produced by the parent cultures.

This paper reports experiments to determine if common bacterial food contaminants of public health significance could, with repeated exposure, develop greater resistance to gamma irradiation.

Materials and Methods

Apparatus, dosimetry, irradiations from a Co^{60} source, and analytical methods were as outlined in a previous paper (2).

Cultures of *Staphylococcus aureus*, *Salmonella gallinarum*, *Escherichia coli*, and *Streptococcus faecalis* were grown and irradiated under aerobic conditions in nutrient broth (Difco) containing 0.3% yeast extract. After incubation for 20 hours at 35.5° C broth cultures were irradiated at dosages calculated from preliminary experiments to produce over 99% mortality in the parent cultures. Immediately following irradiation, 1 ml of the broth suspension was transferred to fresh broth and after 22 hours incubation at 35.5° C the cycle was repeated until the desired number of exposures had been reached. Resistance development was then measured by simultaneously determining survival at various dose levels for the irradiated culture and the parent culture which had received identical treatment except for lack of irradiation.

The cultures of *Clostridium botulinum* used in this study included a toxigenic strain of type A and of type E and a non-toxigenic variant of type E. Development of induced resistance was attempted by irradiation of cultures in the vegetative state only. Cultures were grown in a broth formulated from the agar medium proposed by Wynne *et al.* (13) but omitting the agar. Aliquots of test suspensions were tested after irradiations by heating (80° C for 10 minutes for type A and 60° C for 15 minutes for type E) to ensure that they remained spore-free. The culture and irradiation sequence followed the pattern adopted for the non-sporing aerobes except that the *C. botulinum* cultures were grown and irradiated under anaerobic conditions in yeast extract starch bicarbonate broth.

Low temperature irradiations were carried out by freezing the cultures in irradiation vials in an acetone – dry-ice bath immediately after filling. They were then held in crushed dry ice (−78° C) during subsequent handling and irradiation and were thawed in a water bath at 40° C just prior to analysis.

Owing to the generally exponential nature of radiation survival curves, plots of percentage survival (logarithmic scale) versus radiation dose (arithmetic scale) have been chosen as the most convenient way of expressing survival data.

Results

No change in radiation sensitivity could be detected in *Salmonella gallinarum* after 14 consecutive irradiations at 200,000 r.e.p., nor in the reactions normally used in the isolation and identification of salmonellae. Similarly, two of the three strains of *Staphylococcus aureus* used showed no change in sensitivity after 14 irradiations at 200,000 r.e.p. The unchanged strains were isolated, respectively, from pastry involved in a food-poisoning episode and from cheese. The third, a "hospital" strain, showed a moderate increase in resistance after similar treatment, as seen in Fig. 1. After treatment it took 1.24 times the dose necessary for the parent to reduce survival to the 0.01% level. All strains remained mannitol-positive and coagulase-positive. However, two of them, the food-poisoning and "hospital" strains, showed a large number of mutations from yellow to white colonies on plating after irradiation. Changes in phage patterns were noted, and are listed in Table I.

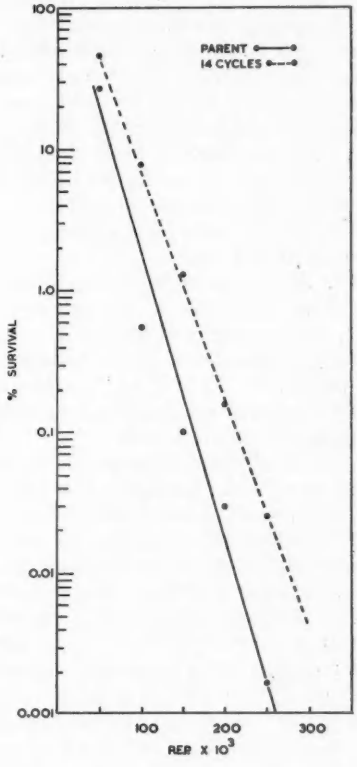


FIG. 1. Change in the radiation sensitivity of *Staphylococcus aureus* after 14 cycles at 200,000 r.e.p.

TABLE I

Phage patterns of parent and irradiated strains of staphylococci

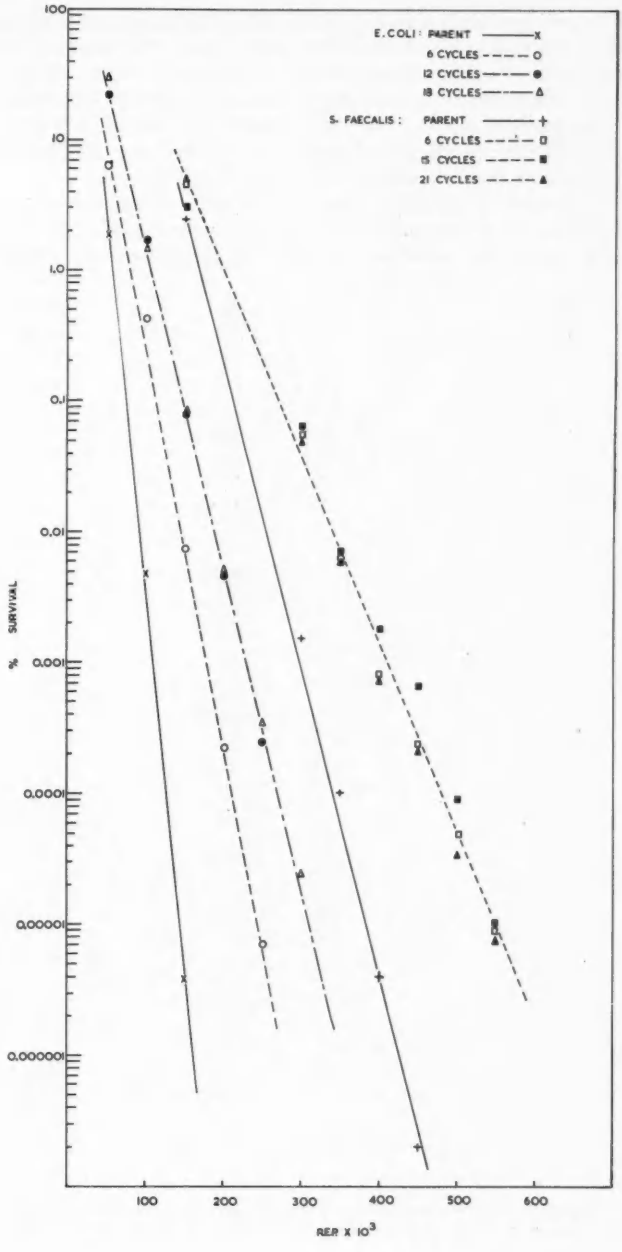
Strain and source	Treatment	Phage patterns
CS 68 cheese isolate	Parent	81 + (7, 54, 75 ±)
	14 cycles	81
ML 15 food-poisoning isolate	Parent (yellow)	6/7/47/53/54/75/81
	14 cycle (yellow)	6/7/47/53/54/75/81
	14 cycle (white)	6/7/47/53/54/75/81
584600 hospital isolate	Parent	80/81
	14 cycle (yellow)	(52)/52A/80/81
	14 cycle (white)	52/52A/80/81

Figure 2 shows the development of irradiation resistance by *S. faecalis* and *E. coli* following the cyclic irradiation treatment at 200,000 r.e.p. for *S. faecalis* and 150,000 r.e.p. for the more sensitive *E. coli*. The development of resistance occurred rapidly with *S. faecalis*, reaching a maximum within 6 consecutive irradiations, then remaining constant through a further 15 irradiations. *E. coli* showed a slower response, reaching a maximum only after 12 cycles and showing no further increase in resistance after an additional 6 cycles. However, *E. coli* developed a higher degree of resistance to irradiation than did *S. faecalis*. In order to reduce survival to the 0.0001% level the resistant cultures of *E. coli* took 1.9 times and *S. faecalis* took 1.6 times the irradiation dosages necessary to obtain the same survival with their parent cultures. The resistant mutants of both species appear stable, no change in resistance being evident after 8 months' maintenance on standard media.

The resistant culture of *S. faecalis* showed no differences from the parent in colonial appearance or in its reactions to the tolerance tests (growth in the presence of 6.5% NaCl, sodium azide, at pH 9.6 and after exposure to 60° C for 30 minutes) which are used in enterococcal detection. Growth levels reached by the resistant strain in broth at 35.5° C for 24 hours were very slightly less than that attained by the parent strain, levels reached being 19×10^7 and 25×10^7 cells per ml, respectively.

Isolates from the irradiated *E. coli* culture showed no change in methyl red, V.P., citrate, or gelatin reactions. However, a change did occur in indol production. After 6 irradiations some indol-positive cultures could be recovered, but after 12 irradiations, all isolates were indol-negative. When plated on EMB agar immediately after irradiation most colonies produced no sheen although with subsequent transfers this property was considerably regained. Colonies which produced sheen, and dark colonies with no sheen fermented lactose in both EC broth at 44.5° C and lactose broth at 37° C; dark pink colonies with no sheen failed to ferment lactose at 44.5° C and small light pink colonies with no sheen failed to ferment lactose in 3 days at both 44.5° C and 37° C. The primary change, based on Imvic characteristics, seems

FIG. 2. Changes in the radiation sensitivities of *Escherichia coli* cycled at 150,000 r.e.p. and *Streptococcus faecalis* cycled at 200,000 r.e.p.


E. COLI: PARENT
6 CYCLES
12 CYCLES
18 CYCLES
S. FAECALIS: PARENT
6 CYCLES
15 CYCLES
21 CYCLES
% SURVIVAL
100
10
1.0
0.1
0.01
0.001
0.0001
0.00001
0.000001
100
200
300
400
500
600
RER X 10³

to be from *E. coli* I to Irregular II and *E. coli* II, although a few lactose negative colonies were also isolated. However, no attempt has been made to determine the frequency of occurrence of the various mutants. The radiation tolerance of all isolated mutants was of the same order. No mutants were detected in the parent culture. The parent strain in broth at 35.5° C multiplied to a level of 10^8 cells per ml in 24 hours. The irradiation cycled culture under the same conditions reached a level of 1.2×10^9 cells per ml.

Type E strains of *Clostridium botulinum* rapidly mutated to the non-toxigenic, sporulating phase whether being irradiated or not, and since maintenance of a spore-free state was extremely difficult, no results are presented for type

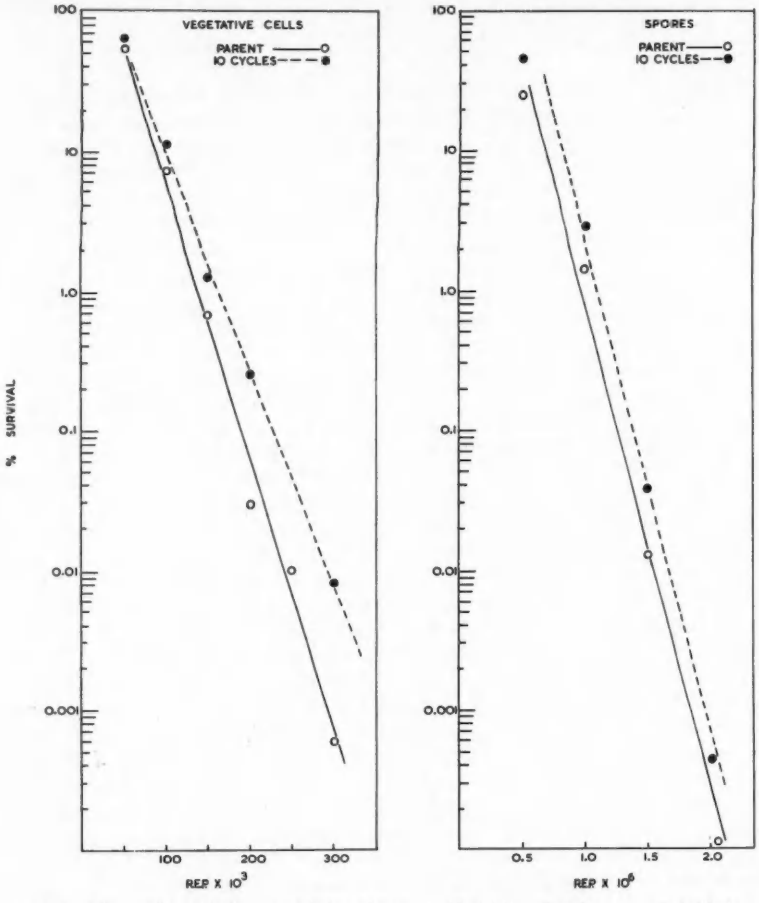


FIG. 3. Change in radiation sensitivity of type A *Clostridium botulinum* cycled 10 times at 200,000 r.e.p. in the vegetative state.

E strains. However, sporulation could not be demonstrated during daily transfer and irradiation of the type A strain, and Fig. 3 illustrates the increased radiation resistance developed by vegetative cells subjected to 10 consecutive daily irradiations of 200,000 r.e.p. The resistant strain at this point took 1.3 times the dose necessary to reduce the parent strain to the same percentage

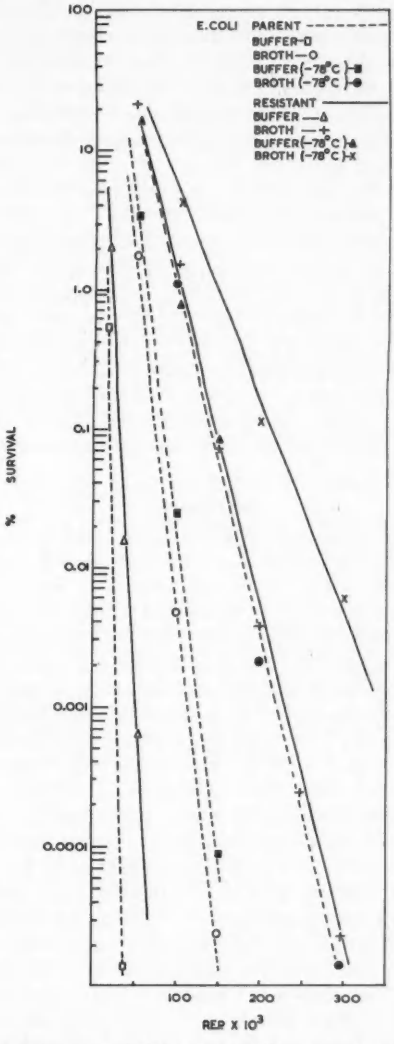


FIG. 4. Effect of irradiation at −78° C and at 5° C on parent and "resistant mutant" strains of *E. coli* suspended in buffer and in broth.

(0.01%) survival. Subsequently the culture was allowed to sporulate and the radiation resistance of these spores was compared to that of the spores of the parent culture. The data, illustrated in Fig. 3, show a constant slightly greater survival for the spores of the resistant culture at all radiation levels tested. The increased resistance to irradiation was transmitted through the spore stage to the subsequently grown vegetative cells.

Concurrently with radiation resistance tests, these cultures were also tested for toxin production. The original type A culture produced approximately 4×10^6 mouse M.L.D. of toxin per ml and the culture receiving 10 irradiations showed no decrease, while a duplicate culture handled in a manner identical with that of the irradiated one but receiving no irradiation produced only 5×10^5 mouse M.L.D. of toxin per ml.

Figure 4 illustrates the comparative lethality of irradiation at −78° C and 5° C on parent and "induced resistant" cultures of *E. coli* suspended in broth and in buffer. In all cases irradiation at −78° C provided increased protection to the cultures used. However, in comparing irradiation dosages necessary to reduce survival to the 0.0001% level the "induced resistant" strain always needed 1.9 times the dose for the parent strain under identical irradiating conditions. In addition the survival curves for both the parent and resistant strains when irradiated in buffer suspension at −78° C were almost identical with the curves from the same cultures irradiated in broth at 5° C.

Similarly, the comparative differences in sensitivity between parent and resistant strains of *S. faecalis* remained constant during irradiation at 5° C and at −78° C.

Discussion

The data presented show that induced resistance to irradiation can be developed by specific bacteria. Resistance increased in a stepwise fashion during multiple irradiations, finally reaching a "plateau" beyond which no further resistance to a constant dose could be demonstrated. The extent and rapidity of this resistance development varied with bacterial strains and species. The greatest change occurred with *E. coli* where an approximate doubling in irradiation dosage became necessary to reduce a population of 10^6 cells per ml to 1 cell per ml. It was also with this culture that significant changes occurred in biochemical reactions. The reported changes in radiation sensitivity were developed by irradiation at a constant dose for each species. Whether or not the increased tolerance could be extended by subjecting the "resistant" variants to higher doses is currently under investigation.

Recent emphasis placed on physical and chemical changes occurring in the whole irradiated environment (5, 10) suggested that the induced resistance observed might be a resistance to the toxic effects of reaction products from free-radicals produced during irradiation. Freezing might be expected to extend protection from ionizing radiations by limiting the mobility of the free-radicals produced and this in fact has been reported (1, 3, 6). If the demonstrated "induced resistance" expressed a resistance to toxic by-products produced during irradiation, then the irradiation of parent and "resistant" strains in the frozen state could be expected to reduce the differences in the respective survival curves. The fact that this did not occur, and that differences between

parent and resistant strains remained constant when irradiated in broth or buffer suspensions at −78° C or 5° C lends weight to the concept that the observed resistance does not express resistance to the reaction products formed within the medium during irradiation.

The significance of the development of radiation resistant mutants is of obvious importance in the radiation preservation of foods, particularly for application of so-called "pasteurising doses" where the margin of tolerance for a dose that will destroy spoilage bacteria without causing adverse organoleptic changes may be small. Although only a doubling has been shown in the resistance of *E. coli*, this may result, at a specific treatment dose, in a large increase in the number of surviving organisms some of which may be mutants no longer recognizable as of fecal origin.

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MALIC ENZYME INDUCTION BY LACTIC ACID BACTERIA

II. PURINE AND PYRIMIDINE REQUIREMENTS[1]

JOHN A. DUERRE AND HERMAN C. LICHSTEIN

Abstract

Malic enzyme activity was shown to be inducible in cells of *Lactobacillus arabinosus*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Leuconostoc mesenteroides*, and *Streptococcus faecalis*.

Inspection of the nitrogen requirements for induction revealed an obligatory need for purines and uracil by *L. casei* and *L. delbrueckii*. Addition of purines and uracil also stimulated induction by *S. faecalis* and *L. arabinosus*, but not by *L. mesenteroides*. The role of purines was inspected further by the inhibitory action of 8-azaguanine.

Studies on protein and RNA synthesis during malic enzyme induction revealed that RNA synthesis preceded protein synthesis. This phenomenon was masked in the absence of added purines and uracil by concomitant degradation of cellular RNA. Such breakdown of RNA was observed also when the cells were aged at pH 7.

The stimulatory effect of biotin on malic enzyme induction by *L. arabinosus* could be replaced partially by adenine, and the inhibitory effect of homobiotin was reversed by the addition of this purine. The absence of a biotin effect on induction by *L. casei* may be related to the requirement for purines and uracil in this organism.

Introduction

Deal and Lichstein (4) have suggested the amenability of enzyme synthesis studies as a measure of nutritional interactions. Using washed bacterial suspensions of *Lactobacillus arabinosus* 17-5, they demonstrated that the nutritional requirements for malic enzyme synthesis closely paralleled those necessary for growth.

The present report extends such studies to taxonomically related microorganisms, some of which are nutritionally more exacting than *L. arabinosus*.

Methods

L. arabinosus 17-5 (ATCC 8014), *Lactobacillus delbrueckii*, *Lactobacillus casei* (ATCC 7469), *Leuconostoc mesenteroides* P-60, and *Streptococcus faecalis* R (ATCC 8043) were studied and were maintained in stock culture by monthly transfers on APT agar (Case Laboratories, Chicago, Ill.). The experimental medium was that designed by Wright and Skeggs (12). Incubation and harvesting of the cultures were done according to procedures already outlined (4), except that *L. casei* and *L. delbrueckii* required an incubation period of 21–23 hours.

The rate of malic enzyme formation was determined by manometric measurement of CO_2 evolution from L-malate. The nutrients added to the induction system were the same as those employed previously (4) with the exceptions as noted in the particular experiment.

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In preparation for ribonucleic acid (RNA) determinations on bacterial cells from induction studies, the contents of the experimental vessel were boiled for 5 minutes to stop enzyme activity, then subjected to centrifugation at 17,000 × *g* for 20 minutes in the cold, and reconstituted with distilled water to the original volume of cells added. Since a minimum of 5 ml of the original cell suspension was required for RNA and protein determinations. 50 ml screw-capped tubes were employed as the experimental vessel and contained 10 times the volume of a Warburg vessel with all conditions being identical with those usually employed. The supernates were recovered after centrifugation for the determination of residual purines and pyrimidines, which were measured at 260 mμ (Beckman DU spectrophotometer). Ribose, derived from RNA by trichloroacetic acid hydrolysis of the bacterial cells, was determined by the orcinol method of Mejbaum (10). Acid soluble pentoses were removed prior to hydrolysis by treatment of the cells in a Potter–Elvehjem homogenizer and washing 3 times with cold 5% trichloroacetic acid. Since hexoses interfere with the determination of pentoses (2), all samples were corrected for reactive ribose by determining the % density due to purified yeast RNA in the presence of a known concentration of glucose. The corrected density was compared to a ribose standard, and calculations were based on a tetranucleotide with the ratio of purines to pyrimidines approximating one. Because of the inability of hot trichloroacetic acid to hydrolyze pyrimidine nucleotides, reactive ribose was multiplied by two to give RNA ribose (7). The colorimetric method of Johnson (8) was employed for the determination of total cell protein. Protein was estimated by multiplying the nitrogen value by 6.25.

Results and Discussion

Initial studies were undertaken to determine if the inducible malic enzyme of *L. arabinosus* (1) was present and inducible in other taxonomically related microorganisms. The inductive nature of the enzyme was established by comparing malic enzyme activity of bacterial cells grown in Wright–Skeggs medium with and without the addition of malate. Preinduced cells (cultured with malate) exhibited a linear response to malate without demonstrable lag (Fig. 1). In contrast, cells grown in the absence of malate and tested under appropriate conditions revealed a definite increase in activity after a lag period which varied considerably among the organisms inspected. Although not presented in the figure, *S. faecalis* and *L. mesenteroides* exhibited induction patterns quite similar to *L. arabinosus*, whereas *L. delbrueckii* was similar to *L. casei* in this respect.

Formation of an induced enzyme rather than selection of a mutant was established in several ways. For example, subculturing the induced cells in media lacking malate revealed that none of the organisms exhibited preexisting enzyme after a single transfer. Furthermore, enzyme formation was blocked completely by the addition of chloramphenicol (100 μg) or by exposure of the bacterial cells to ultraviolet rays (approximately 2537 Å). Such treatments had no effect on the malic enzyme activity in preinduced cells.

Attention was directed next to the nutritional requirements for malic enzyme induction, and among these the nitrogen requirements were inspected first. Of considerable importance was the finding that a mixture of amino

acids (4) supported excellent enzyme induction by *L. arabinosus*, *L. mesenteroides*, and *S. faecalis*, whereas *L. casei* and *L. delbrueckii* failed to carry out significant induction under these conditions (depicted for *L. arabinosus* and *L. casei* in Fig. 2). The further addition of the purines, adenine, guanine, and

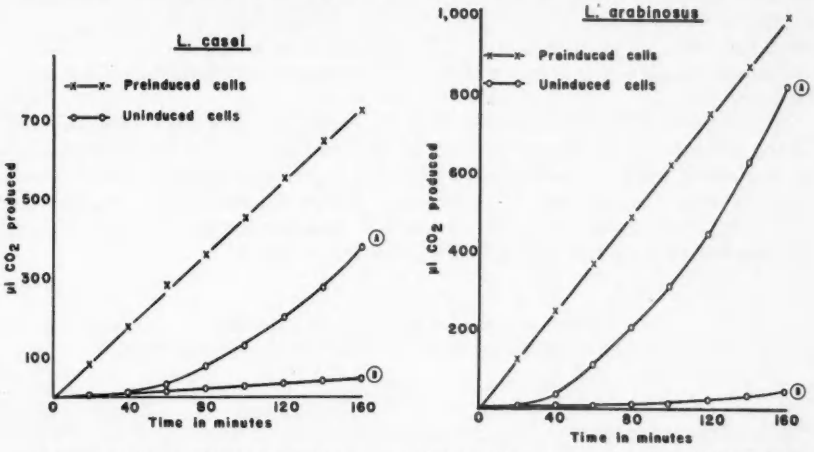

FIG. 1. Evidence for the inductive nature of the malic enzyme in lactic acid bacteria. A = uninduced cells with Wright–Skeggs medium (0.5 ml double strength) and glucose (0.08 *M*) added to the Warburg flask; B = uninduced cells with medium and (or) glucose omitted from the Warburg flask.

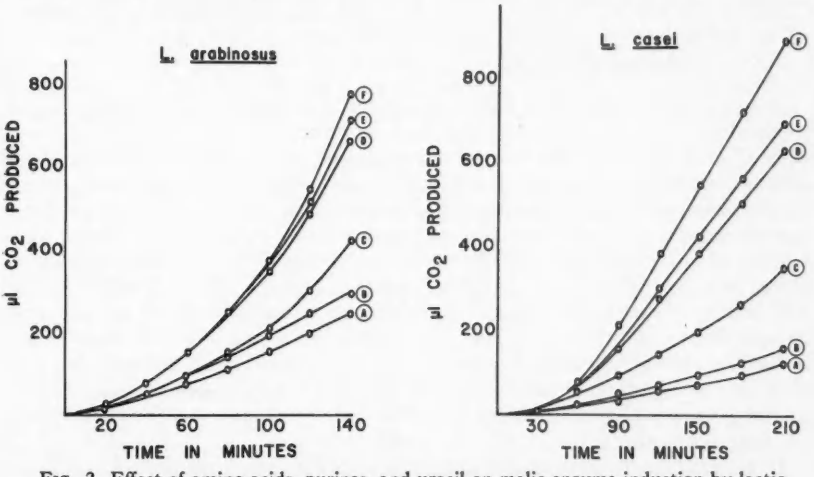

FIG. 2. Effect of amino acids, purines, and uracil on malic enzyme induction by lactic acid bacteria. A = amino acid mixture (0.5 ml); B = amino acid mixture + adenine, guanine, xanthine, uracil (0.1 μg each); C = as B except 1 μg each of purines and uracil; D = as B except 10 μg each of purines and uracil; E = as D plus biotin, pyridoxal, PAB, thiamine, nicotinic acid, and pantothenate (conc. as found in 0.5 ml double strength Wright–Skeggs medium); F = Wright–Skeggs medium (0.5 ml double strength).

xanthine, and the pyrimidine, uracil, to the induction system permitted significant malic enzyme synthesis by *L. delbrueckii* and *L. casei* (shown for the latter organism in Fig. 2). Moreover, these compounds also increased the level of induction by *L. arabinosus* and *S. faecalis*, while having no effect on *L. mesenteroides*. Thus, of the organisms tested, *L. casei* and *L. delbrueckii* exhibited an absolute requirement for these compounds. It is noted (Fig. 2) that the addition of a mixture of B vitamins was without effect, probably reflecting the fact that the organisms were not grown deficient in these compounds.

A titration of the individual purines and uracil made known that any one of the purines tested (50 μg) permitted maximum induction by *L. arabinosus* or *S. faecalis*, whereas the further addition of uracil was required for optimum induction by *L. casei* and *L. delbrueckii* (Table I). Although cytosine and thymine could replace partially the uracil requirement for *L. casei* and *L. delbrueckii*, they had no effect in the presence of uracil.

TABLE I

Effect of purines on malic enzyme induction

Additions	Q_{CO_2}	
	L. casei	*L. delbrueckii*
None	42	75
Uracil + adenine	306	360
Uracil + guanine	302	370
Uracil + xanthine	312	355
Uracil + adenine + guanine + xanthine	310	450
Adenine + guanine + xanthine	152	270

NOTE: Purines and pyrimidine added at a level of 20 μg/flask.
Nitrogen source was amino acid mixture.
Q_{CO_2} based on final 20-minute period.

A study of compounds known to inhibit the metabolism of purines and pyrimidines revealed that 8-azaguanine was the most effective. The results with *L. arabinosus* (Table II) showed complete inhibition of induction in the absence of added purines and uracil by a concentration of 10 μg of 8-azaguanine. In the presence of purines and uracil, a concentration of 500 μg of the inhibitor was necessary, and the inhibition could be reversed by increasing the concentration of any one of the purines. With *L. delbrueckii*, an organism having an obligatory requirement for purines and uracil, induction was inhibited by 100–500 μg of 8-azaguanine, and the inhibition could be reversed significantly by guanine or xanthine, but not by adenine. It appears that *L. delbrueckii*, unlike *L. arabinosus*, cannot convert adenine to guanine. However, this conclusion is not in keeping with the data in Table I, which showed that adenine in the presence of uracil supported induction by *L. delbrueckii*, albeit not at an optimum rate.

It is pertinent to note that 8-azaguanine had no effect on preinduced malic enzyme activity and, moreover, when introduced during enzyme induction (after 60–80 minutes) further enzyme formation was prevented. Although the site of action of 8-azaguanine is not elucidated from these data, it appears

that it is not primarily concerned with preventing the entrance of purines into the bacterial cell, since the inhibitor was very effective even in the absence of added purines.

TABLE II

Inhibition of malic enzyme synthesis by 8-azaguanine

Additions to mixture of 18 amino acids	Conc. of 8-azaguanine, μg/flask	Q_{CO_2}	
		L. arabinosus	*L. delbrueckii*
None	0	355	58
	0.1	348	
	0.5	200	
	1.0	140	
	10.0	12	
Purines + uracil*	0	640	430
	1.0	635	400
	10.0	505	230
	100.0	140	30
	500.0	70	0
Purines + uracil + guanine (500 μg)†	100.0	630	340
Purines + uracil + adenine (500 μg)†	100.0	500	90
Purines + uracil + xanthine (500 μg)†	100.0	430	250

NOTE: Q_{CO_2} based on final 20-minute period.
*10 μg/flask of adenine, guanine, xanthine, uracil.
†Other purines and uracil, 10 μg/flask.

Since purines and uracil exerted such a marked effect on malic enzyme induction, it was of interest to correlate the amount of RNA and protein formed during the course of induction. From the data presented (Table III), it is apparent that *L. arabinosus* synthesized considerable quantities of cellular protein and RNA in the absence of inducer. This was accompanied by a 100% increase in bacterial cell population, as determined both by viable

TABLE III

RNA, protein, and malic enzyme synthesis by *L. arabinosus*

Ingredients in Warburg flask in addition to cells and buffer	Total protein, μg	RNA, μg	RNA/ protein	Malic enzyme activity, Q_{CO_2}
None (zero time control)	610	106	0.173	—
None	560	87	0.155	0
Salts	585	97	0.165	0
Salts, a.a., glucose	900	119	0.133	0
Salts, a.a., glucose, malate	990	124	0.125	530
Salts, a.a., glucose, P.P.	900	147	0.163	0
Salts, a.a., glucose, P.P., malate	1380	247	0.179	810
Salts, glucose, P.P.	585	117	0.200	0
Salts, glucose, P.P., malate	615	104	0.169	72
Salts, glucose, a.a., P.P., 8AG	724	130	0.179	0
Salts, glucose, a.a., P.P., malate, 8AG	820	144	0.176	25

NOTE: a.a. =amino acid mixture; P.P. =adenine, guanine, xanthine, uracil (10 μg each); 8AG =8-azaguanine (500 μg); incubation time, 3 hours.

cell counts and dry weight. In the presence of inducer, a slight additional increase in both protein and RNA was noted, and the further addition of purines and uracil resulted in a marked increase in the synthesis of these components. This increase could not be attributed solely to the synthesis of malic enzyme since cell population increased by 300%. However, the amount of protein synthesized in the presence of purines and uracil was 30% greater than the amount produced in their absence and, concomitantly, malic enzyme activity increased by 70%. This finding suggests that the formation of new protoplasm was accelerated by the presence of purines and uracil and that a greater percentage of this synthesis was directed toward the production of the malic enzyme system. Such observations might account for the excellent correlation between the factors required for malic enzyme synthesis and those needed for growth. Of further interest was the finding, in agreement with those of other workers (5), that a mixture of 18 amino acids was essential for RNA synthesis.

It is pertinent also (Table III) that whereas 8-azaguanine prevented the formation of malic enzyme, some protein and RNA synthesis did occur.

Also of significance was the finding (Table III) that breakdown of pre-existing RNA occurred in control cells incubated for 3 hours in phosphate buffer (pH 5.5). Such breakdown could not be attributed solely to cell lysis, since total protein did not diminish appreciably. Further examination revealed that cells of *L. arabinosus* subjected to aging in 0.2 *M* phosphate buffer (pH 7.0) lost large quantities of purines and pyrimidines to the surrounding menstruum (Fig. 3). These results are in keeping with those of Holden (6). Chemical analysis of this supernatant fraction also disclosed the presence of pentose sugars. Perhaps of more importance was the finding that the aged bacterial cells contained high levels of intracellular acid soluble pentose which may be involved in the synthesis of new RNA.

In view of these results, experiments were designed to determine the amount of RNA and protein synthesized during a complete enzyme induction period.

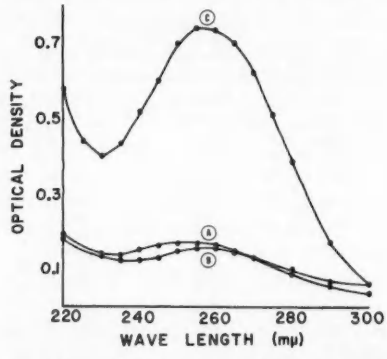

FIG. 3. Spectrophotometric analysis of supernates collected from cells of *L. arabinosus* aged for 30 minutes at 37° C in distilled water (curve A), 0.2 *M* phosphate buffer, pH 4.0 (curve B), and 0.2 *M* phosphate buffer, pH 7.0 (curve C).

It is seen (Fig. 4) that protein and RNA breakdown occurred in the absence of added purines and uracil during the first 20 minutes of induction, after which an increase in the synthesis of these compounds by *L. arabinosus* was noted accompanied by increased malic enzyme activity. When purines and uracil were added to the induction system, no degradation of pre-existing RNA was noted, and a sharp increase in RNA synthesis was apparent after a 20-minute lag period. Total protein and malic enzyme activity increased about 10 minutes after RNA synthesis started, thus agreeing with the concept that RNA synthesis precedes protein synthesis (3, 9). This effect was not apparent when purines and uracil were deleted from the induction system, but turnover of pre-existing RNA could be occurring, thus masking an increase

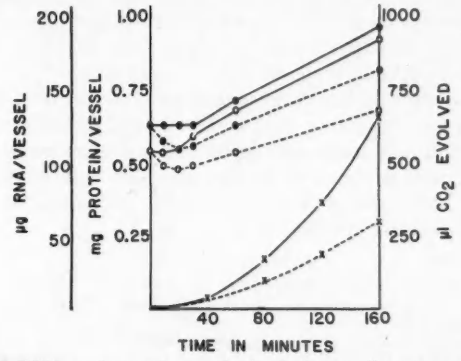

FIG. 4. Rate of RNA and protein synthesis during malic enzyme induction by *L. arabinosus*. Source of nitrogen was amino acid mixture; 0.85 mg dry wt. of bacterial cells/Warburg flask. – – – Without purines and uracil. ——— Plus adenine, guanine, xanthine, uracil (10 μg each). ○ RNA. ● Protein. × CO_2 production.

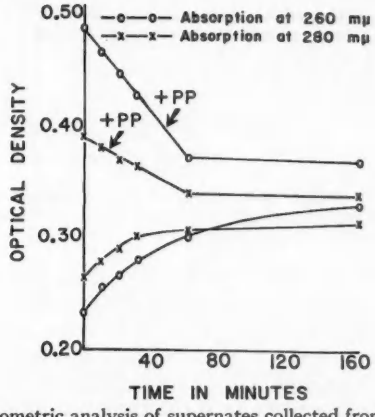

FIG. 5. Spectrophotometric analysis of supernates collected from cells of *L. arabinosus* during malic enzyme induction. PP=addition of adenine, guanine, xanthine, and uracil (10 μg each). Other conditions as for Fig. 4.

in net RNA synthesized. That such turnover was indeed taking place even in the presence of net RNA synthesis was substantiated by spectrophotometric analysis of the supernates collected from the bacterial cells during the course of induction (Fig. 5). It is manifest that there was an increase in the 260 mμ absorbing fraction up to 80 minutes after initiation of induction. The leakage of 280 mμ absorbing materials, presumably aromatic amino acids, apparently stopped after 30 minutes. This corroborates the previous finding that cell death and lysis were not contributing substantially to the purine–pyrimidine pool. On the other hand, this loss did not occur in the presence of added purines and uracil. After 60 minutes the cells had presumably become capable of synthesizing their own supply of purines and pyrimidines or had accumulated sufficient quantities in the intracellular pool to support induction.

Similar experiments with *L. casei*, which exhibits a much longer lag period prior to induction, revealed more strikingly the fact that RNA synthesis preceded protein synthesis (Fig. 6). The nature of the curve obtained when purines and uracil were added suggest that the inducer was primarily responsible for the initial increase in RNA. The additional rise in RNA synthesis after 2 hours, which was accompanied by a sharp increase in protein, suggests that cell growth and division were occurring at this time. This point was clarified further by viable cell counts which indicated an increase in cell numbers after 2 hours.

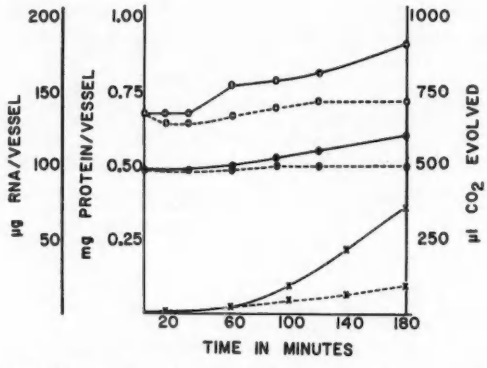

FIG. 6. Rate of RNA and protein synthesis during malic enzyme induction by *L. casei*. Notations on figure identical with those of Fig. 4. Conditions as for Fig. 4 except 1.1 mg dry wt. of bacterial cells/Warburg flask.

Finally, because of the role of biotin in purine biosynthesis (11), and in view of the effect of purines on the malic enzyme system, it was of interest to investigate this aspect in *L. arabinosus* and *L. casei*. A requirement for biotin during growth was manifest, with the assayable range being approximately the same for both organisms (10^{-4}–10^{-2} μg/10 ml). A requirement for this vitamin for malic enzyme induction was observed for *L. arabinosus* but not *L. casei*. Furthermore, the addition of homobiotin had no effect on induction by cells of *L. casei*.

Further work with *L. arabinosus* revealed that adenine was capable of replacing in significant measure the biotin stimulation of malic enzyme induction (Fig. 7). Moreover, adenine completely overcame the inhibitory effect of homobiotin (Fig. 8). Such findings suggest that an important function of biotin in the malic enzyme system is via its yet unresolved role in the formation of purines. This suggestion was strengthened by the finding that *L.*

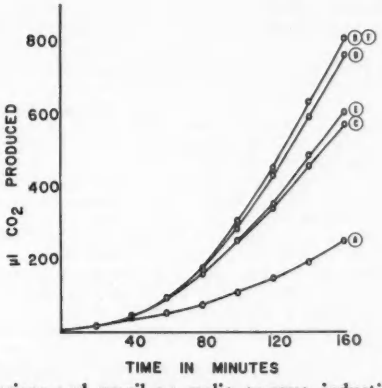

FIG. 7. Effect of purines and uracil on malic enzyme induction by biotin-deficient cells of *L. arabinosus*. Cells harvested from Wright–Skeggs medium containing 4×10^{-6} μg biotin/ml. All Warburg flasks contained amino acid mixture as source of nitrogen. A = control; B = plus biotin (10^{-3} μg); C = plus adenine, guanine, xanthine, uracil (10 μg each); D = biotin (10^{-2} μg) + purines and uracil as in C; E = plus adenine (10 μg); F = adenine (10 μg) + biotin (10^{-2} μg).

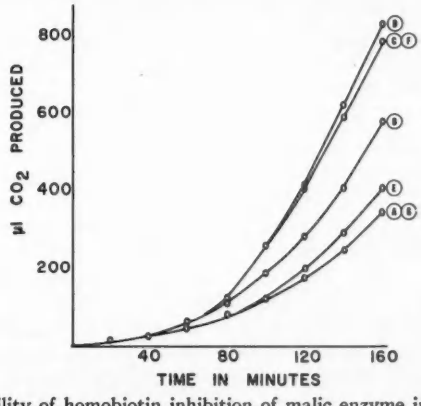

FIG. 8. Reversibility of homobiotin inhibition of malic enzyme induction by adenine in biotin-deficient cells of *L. arabinosus*. Conditions as for Fig. 7. A = control; B = plus adenine (10 μg); C = plus biotin (10^{-2} μg); D = adenine (10 μg) + biotin (10^{-2} μg); E = as C + homobiotin (50 μg); F = as E + adenine (10 μg); G = as C + homobiotin (500 μg) with or without adenine (10–100 μg). The homobiotin was kindly supplied by Hoffmann-LaRoche Inc.

arabinosus did not synthesize RNA in the absence of biotin. However, turnover in pre-existing RNA appeared to take place, which may account for the limited amount of malic enzyme synthesized in the absence of biotin. Moreover, since *L. casei* exhibits an obligatory requirement for purines and uracil and no requirement for biotin, the possibility that biotin might be involved in the biosynthesis of one or more of these compounds appears to be obviated in this case.

The sparing effect of aspartic acid on biotin stimulation of malic enzyme synthesis (4) suggests a dual role for biotin in this system.

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THE METABOLISM OF GIBBERELLA FUJIKUROI IN STIRRED CULTURE[1]

A. Borrow, E. G. Jefferys, R. H. J. Kessell, Eithne C. Lloyd, P. B. Lloyd, and I. S. Nixon

Abstract

The growth of *Gibberella fujikuroi* in stirred culture was studied. Media were designed in which the initial concentrations of glucose (G), nitrogen (N), phosphorus (P), and magnesium (M) were varied so that the first nutrient was exhausted at a selected dry weight, and the remaining nutrients in all available sequences thereafter. Distinct phases of growth, related to the changing nutritional status of the mold, have been defined.

The *balanced phase* was a period of proliferation in the presence of all nutrients, during which a unit increase in dry mycelium was accompanied by constant uptakes of G, N, P, M, and potassium (K). The morphology remained virtually unchanged, and the dry mycelium contained ca. 6% fat, ca. 16% carbohydrate, and ca. 13% phosphorus-containing compounds, and the fractions of each remained constant. This phase continued until the first nutrient was exhausted. Proliferation, indicated by increases in fat- and carbohydrate-free dry weight, then ceased in N- and G-limited fermentations. In P- and M-limited fermentations, proliferation continued until the subsequent exhaustion of either G or N, and during this *transition phase* the carbohydrate content of the mycelium increased, while in M-limited fermentations the fat content also increased. In P-limited fermentations the uptake of both M and K ceased, and reserves of metaphosphate were used.

At the exhaustion of N in N-, P-, or M-limited fermentations, proliferation ceased but the dry weight continued to increase due to increases in carbohydrate and triglyceride. In the presence of sufficient glucose, maximum amounts of 45% fat, visible as oil globules, and 32% carbohydrate were formed, but in the presence of less G these ceased to increase when G was exhausted before these maxima were reached. In either case the *maintenance phase* followed, during which all mycelial components remained constant except the triglycerides, which decreased when exhaustion of G had initiated the maintenance phase; otherwise they also remained constant until G was subsequently exhausted, and then decreased. The *terminal phase* began when the triglycerides were exhausted, and also when G was exhausted in G-limited fermentations, or in P- and M-limited fermentations in which G was exhausted before N. This phase was characterized by hyphal breakdown, a decrease in dry weight, and the liberation of mycelial components into the medium.

Introduction

Gibberella fujikuroi (Saw.) Wr. (conidial state *Fusarium moniliforme* Sheld.) is of interest because the plant growth regulating substances gibberellic acid and the gibberellins have been isolated from its culture filtrates and because it is the organism responsible for the Bakanae disease of rice. Other compounds isolated from the filtrates include the fusaric acids, succinic acid, and 5-hydroxymethyl-furan-2-carboxylic acid (49).

With the exception of Yabuta, Sumiki, and Uno (60), who studied the effect of varied concentrations of glycerol or glucose, initial pH, and incubation time on the production of gibberellins and fusaric acid in surface culture, few investigations have been published on the growth and metabolism of the mold

[1]Manuscript received January 13, 1961.
Contribution from Imperial Chemical Industries Ltd., Akers Research Laboratories, The Frythe, Welwyn, Hertfordshire, England.

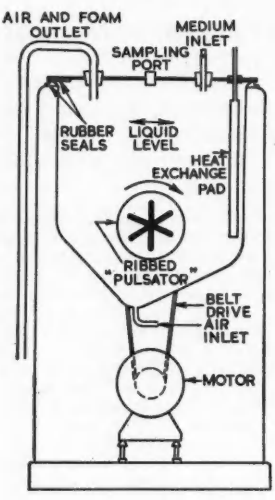


FIG. 1

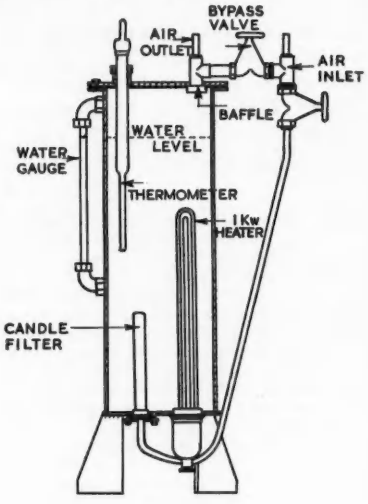


FIG. 2

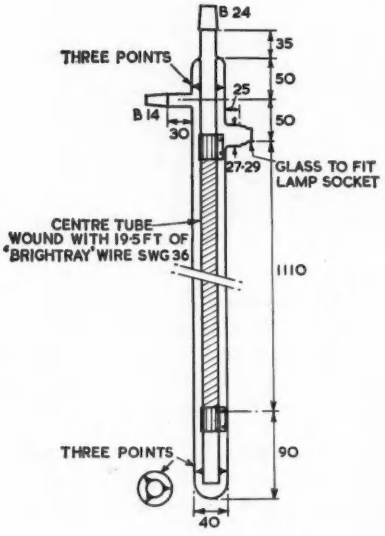


FIG. 3

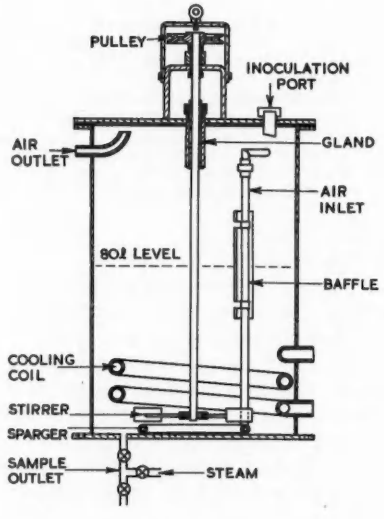


FIG. 4

FIG. 1. 'Hoover' 30-l. fermenter (×0.082).
FIG. 2. Humidifier (×0.082).
FIG. 3. Air sterilizer. Dimensions in millimeters.
FIG. 4. 80-l. fermenter (×0.059).

under varied conditions. Previous work has been done on surface culture (6, 51, 58), in shaken culture (28, 51), and in stirred culture (6, 28, 50) within a limited range of conditions.

Similarly, little work has been done on the composition of the mycelium. Thomas (52) included *Fusarium moniliforme* in his survey of the cell wall of the fusaria. He postulated a laminated structure consisting of a protein–pectic compound, a cellulose – fatty acid complex, and a basic skeleton of chitin. However, Frey (19) failed to confirm the presence of cellulose in other fusaria. Yabuta, Sumiki, Tamura, and Murayama (59) made a general analysis of surface felts of *G. fujikuroi*, and found 44.3% of dry mycelium to be crude protein, 6.7% crude fat, 15.3% crude fiber, 2.1% ash, and 0.6% of a hydrocarbon. They also identified the following mycelial components: ergosterol, carotenoids, palmitic acid, stearic acid, phosphoric acid, oxalic acid, adenine, betaine, and a polysaccharide containing uronic acid, galactose, ketose, and pentose.

The present experiments were undertaken to study the course of stirred fermentation in detail under a wide range of nutritional conditions, with reference to changes in both mycelium and culture filtrate, and to their interrelations.

Materials and Equipment

Fermentation Equipment

'Hoover' 30-l. Fermenter

This was a modified stainless steel 'Hoover'[2] washing machine (Fig. 1). The wringer was removed and the rivet holes filled with stainless steel rivets. A replacement lid carried a stainless steel heat exchange pad through which water at constant temperature was circulated. Holes were provided in the lid for addition of medium, for inoculation and sampling, and for air and foam discharge. Agitation was provided by the circular ribbed 'Pulsator', revolving at ca. 600 r.p.m., in the side of the fermenter.

The air supply was metered, and then humidified to saturation at the temperature of the fermentation, in the equipment shown (Fig. 2). This air was sterilized in the apparatus shown (Fig. 3). At 15 l./minute the air was heated to ca. 250° C and took ca. 5 seconds to pass through the sterilizer.

It was found in preliminary work that when antifoam agents were added there were decreases in the rate of nutrient uptake and of mycelial accumulation. Similar effects were observed with other fungi and for this reason no antifoam agents were used. The air-free volume of the effluent foam was measured at intervals.

Samples were removed by pipette through the sampling port.

80-l. Fermenter

This was of conventional design, made of stainless steel, 20 in. diameter and 29 in. deep (Fig. 4). The two-paddle stirrer was driven at 425 r.p.m. The supply of cooling water was automatically regulated to provide temperature control to ±0.15° C. Air was humidified and sterilized by passage through larger models of the apparatus already described.

[2]Model 0307, series WA, product of Hoover Ltd., Regent Street, London, W.1.

Organism and Inoculation

The provenance of *G. fujikuroi* (Saw.) Wr. strain ACC.917 has already been detailed (6). This is the C.B.S., Baarn, 'Sawada' strain, and is also culture No. NRRL 2633 and Commonwealth Mycological Institute culture No. 58290.

The organism was grown for 14–21 days in 8-oz medicine bottles on isolation medium (excluding rose bengal) (24). For inoculation the mycelium was rubbed off into sterile water, shaken with glass beads, and used at the rate of one bottle to 40 l. of medium. Alternatively, the mycelium from one slope was used to inoculate an aerated 10-l. bottle containing 5 l. medium, and the fermenters were inoculated with 30 ml of the contents of this after 36–48 hours.

The Dry Weight Tube

The dry weight tube is an apparatus in which mycelium from a known weight of original sample can be retained after separation from the culture filtrate, and in which it can be either dried and weighed, or serially extracted under a number of conditions without the need for quantitative transfer of residues. This apparatus has been used in the measurement of mycelial dry weight, fat, and carbohydrate, and also for the fractionation of the carbohydrate and phosphorus-containing compounds in the mycelium.

Apparatus.—The dry weight tube (Fig. 5) consisted of a 3-cm diameter No. 2 sinter disk set near the base of a 50-ml tube fitted with a D 29 stopper. Below the sinter disk, the tube tapered to a B 10 cone which could be closed by a cap. Cap and stopper were fitted to each tube with stainless steel springs and brass chain. The whole sample was received into a tared tube, the basal joint being closed by its cap, and immediately weighed to the nearest milligram. For filtration, the tube was fitted into the removable lid of a side-arm tube

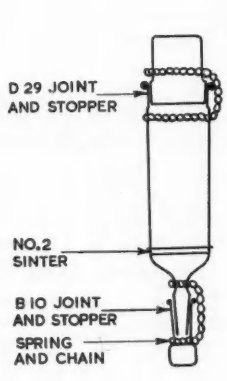


FIG. 5

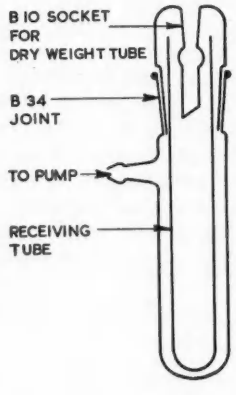


FIG. 6

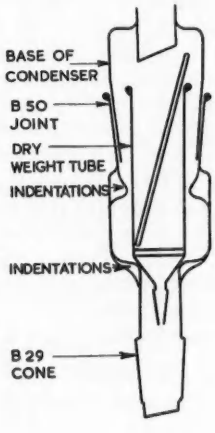


FIG. 7

FIG. 5. Dry weight tube (×0.25).
FIG. 6. Filtration assembly (×0.25).
FIG. 7. Extraction assembly (×0.25).

(Fig. 6). This tube held a 'Pyrex' incineration tube in which the culture filtrate was received and stored.

Hot aqueous extraction of mycelium.—A dry weight tube containing filtered and washed mycelium from a known weight of sample was inserted into the B 10 neck of a 50-ml volumetric flask. The mycelium was suspended in the required reagent, and the stirring rod was left in place. Several such tubes were clipped into a wire basket and immersed in a deep constant-temperature water bath. Expansion of air in the flask below the sinter disk maintained the reagent in contact with the mycelium, while on cooling filtration occurred. Serial extractions and washings could be performed in the same manner, and the pooled filtrates could be made to volume for analysis.

Cold aqueous extraction of mycelium.—The tube, attached to a 50-ml volumetric flask, was immersed in a deep refrigerated bath, and the mycelium was suspended in the required cold reagent. A series of D 29 adaptors accommodating the stirring rods were fitted over the mouths of the tubes, the system was evacuated *in situ*, and filtration took place when the vacuum was released.

Continuous hot solvent extraction.—The tube was placed in the extraction apparatus (Fig. 7), which was then fitted with a receiving flask and condenser. Solvent vapor passed up between the indentations which supported the tube, and the condensate dripped back on to the mycelial pad, returning slowly through the sinter to the receiver.

Experimental

Three experiments are reported, each consisting of a number of fermentations. Unless otherwise stated, all experiments were done in the 30-l. fermenters at 26° C, and air was supplied at 15 l./minute throughout fermentation.

The results of the three experiments are correlated later, but some aspects are described here to explain the design of the later experiments. The methods used to analyze each group of metabolites are given at the start of the relevant sections.

Experiment 1

Sixteen fermentations were studied, in which the initial concentrations of both glucose and NH_4NO_3 were varied. The media, at natural pH, contained initially (g/100 ml) 16, 8, 4, or 2 'Dextrosol'[3] each with 0.96, 0.48, 0.24, or 0.12 NH_4NO_3, together with 0.5 KH_2PO_4, 0.1 $MgSO_4.7H_2O$, and 0.2 (ml/100 ml) minor element concentrate (8). The inocula were derived from agar slope cultures. Samples were removed twice daily, and measurements were made of pH, residual NH_3-N, total inorganic N, glucose, and phosphate; and of the accumulation of mycelial dry weight, fat, and carbohydrate. In some fermentations the mycelial carbohydrate was fractionated.

Glucose- and nitrogen-limited fermentations were distinguished. All other nutrients were in excess. In both types of fermentation growth continued until the time when the limiting nutrient was exhausted, at which time living matter, as measured by the fat- and carbohydrate-free dry weight, reached its maximum concentration. Until either nutrient was exhausted, the proliferating

[3]Glucose monohydrate, a product of Corn Products Ltd., Strand, London, W.C.2.

TABLE I

The composition of media used in experiments 2 and 3

Fig.	Code	g/100 ml								ml/100 ml	
		'Dextrosol'	NH_4NO_3	KH_2PO_4	K_2HPO_4	$K_3PO_4.H_2O$	KCl	K_2SO_4	$MgSO_4.7H_2O$	Minor element	Conc. HCl
Experiment 2											
	G N-m p	7.10	0.605	0.241	0.131	—	—	0.035	0.049	0.2	—
	G N p m	7.10	0.592	0.294	0.099	—	—	0.033	0.052	0.2	—
	G-N P-m	7.10	0.592	—	0.291	0.003	—	0.025	0.063	0.2	0.15
	G n P-m	7.10	0.615	—	0.257	0.033	—	0.025	0.063	0.2	—
8	G M n P	7.10	0.712	0.006	0.279	—	—	0.035	0.049	0.2	—
	G M P n	7.10	0.712	—	0.274	0.011	—	0.034	0.051	0.2	—
	N G p m	9.42	0.552	0.220	0.159	—	—	0.020	0.071	0.2	—
	N G M p	9.42	0.552	0.483	—	—	—	0.034	0.050	0.2	—
	N P G m	11.77	0.552	0.082	0.247	—	—	0.020	0.071	0.2	—
9	N P M G	16.46	0.552	0.105	0.219	—	—	0.034	0.050	0.2	—
	N M P G	20.00	0.552	0.254	0.116	—	—	0.041	0.040	0.2	0.08
	N M G p	11.77	0.552	0.483	—	—	—	0.041	0.040	0.2	—
	P m G N	9.42	0.752	—	0.222	0.056	—	0.034	0.050	0.2	0.08
	P m G n	9.42	1.104	—	0.235	0.039	—	0.041	0.041	0.2	0.10
	P m G n	11.77	1.288	—	0.237	0.037	—	0.041	0.040	0.2	0.12
10	P m N G	20.00	0.832	—	0.237	0.037	—	0.041	0.040	0.2	0.18
	P-m N G	16.46	0.736	—	0.222	0.056	—	0.034	0.050	0.2	—
	P m N G	11.77	0.736	—	0.194	0.094	—	0.020	0.071	0.2	0.08
	M G n p	9.95	0.833	0.354	0.051	—	—	0.043	0.038	0.2	0.03
	M G N p	9.95	0.648	0.309	0.080	—	—	0.043	0.038	0.2	0.07
	M P G n	9.95	1.093	0.146	0.184	—	—	0.043	0.038	0.2	0.11
	M P G n	9.42	0.952	0.035	0.255	—	—	0.043	0.038	0.2	0.13
11	M N P G	10.91	0.648	0.062	0.238	—	—	0.043	0.038	0.2	0.13
	M P N G	13.72	0.732	0.035	0.255	—	—	0.043	0.038	0.2	0.15
Experiment 3											
12	P m N G	12.60	0.500	0.071	—	—	0.112	—	0.100	0.2	—
13	M N G p	14.34	0.611	0.500	—	—	—	0.131	0.005	0.2	—

mycelium was of approximately constant composition, and the uptake of nutrients was directly proportional to the dry weight. These relations made it possible to design media in which chosen concentrations of living matter, and of unused nutrients, would be present at the time of exhaustion of the limiting nutrient.

Experiment 2

This experiment was designed to study the course of nitrogen-, glucose-, phosphate-, and magnesium-limited fermentations (N-, G-, P-, and M-limited) in greater detail than in experiment 1. It was intended that these four nutrients should be exhausted in all possible sequences, and that in each fermentation the first nutrient should be exhausted when the dry weight was 20 mg/g. The initial concentrations of KH_2PO_4, K_2HPO_4, $K_3PO_4.H_2O$, and K_2SO_4 were varied so that constant amounts of potassium and sulphate were supplied; and therefore the initial pH was adjusted when necessary to below 5.5 with HCl prior to autoclaving. Inocula were derived from agar slope cultures.

The media used are shown in Table I, together with a code indicating the sequence of nutrient utilizations found. In this code the letters are arranged in the order of cessation of uptake of these nutrients. A lower case letter indicates that uptake ceased while some of that nutrient remained in the medium; a capital letter, that that nutrient was exhausted. A hyphen between two adjacent letters indicates that these nutrients ceased to be taken up at the same time. In P-limited fermentations the uptake of magnesium ceased soon after phosphate was exhausted. As a result of this unanticipated effect there were only two possible sequences for P-limited fermentations, namely PmNG and PmGN (or n) (Table I). In G-limited fermentations it was also impossible to control the sequence because anabolic activity ceased so soon after the exhaustion of glucose.

All fermentations were sampled at 8-hour intervals, and measurements were made of pH; of residual NH_3-N, total inorganic N, glucose, phosphate, magnesium, and potassium; of the accumulation of mycelial dry weight, fat, carbohydrate, stable acid-soluble phosphorus, acid-labile acid-soluble phosphorus, phospholipid phosphorus, pentosenucleic acid phosphorus, desoxypentosenucleic acid phosphorus, and metaphosphate phosphorus. When sufficient fat was extracted, it was analyzed for its iodine, saponification, and acid values, and the free fatty acid content was calculated. The morphology of the mycelium was also recorded. The course of typical G-, N-, P-, and M-limited fermentations are shown in Figs. 8–11.

In P-limited fermentations the fat- and carbohydrate-free dry weight continued to increase after the exhaustion of phosphate, until such time as either glucose or nitrogen, whichever was first, was in turn exhausted. During this period the acid-insoluble metaphosphate (MP) of the mycelium decreased, while all other phosphorus-containing compounds continued to accumulate. This suggested that MP provided an internal reserve of phosphorus which supported continued growth, but in no fermentation was this reserve exhausted before other external nutrients. Similarly, in M-limited fermentations, the fat- and carbohydrate-free dry weight continued to increase after the exhaustion of magnesium until either glucose or nitrogen was in turn exhausted, and this

suggested that there might also be internal reserves of magnesium. The third experiment was designed to explore these possibilities.

Experiment 3

The media were designed so that either phosphate or magnesium would be exhausted at a relatively low mycelial concentration, in the presence of enough nitrogen to allow mycelial reserves of phosphorus or magnesium to be depleted before nitrogen exhaustion. A further excess of glucose relative to the nitrogen was provided.

Two fermentations were studied, the media for which are shown in Table I. The inocula were grown in an aerated bottle culture, on a medium containing (g/100 ml) 5.0 'Dextrosol', 0.12 NH_4NO_3, and other nutrients as used in experiment 1. Samples were removed and analyzed as in experiment 2. In one fermentation external phosphate was exhausted at a dry weight of 7 mg/g (Fig. 12). In the other, magnesium was exhausted when the dry weight was 5 mg/g (Fig. 13).

Analytical Methods and Results

The Accumulation of Mycelium

Measurement of Mycelial Dry Weight

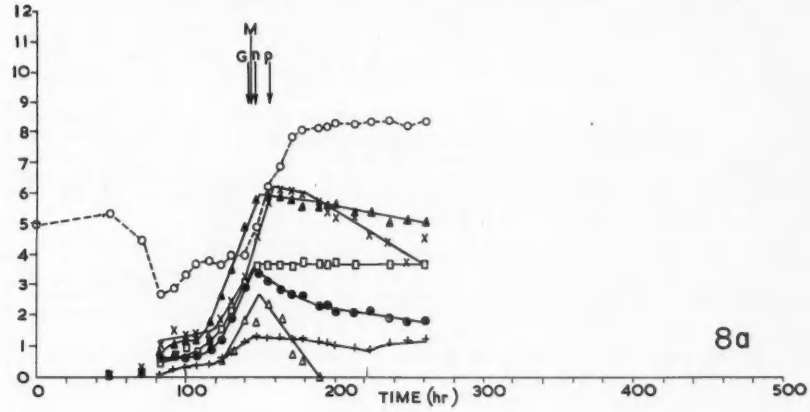
The sample was received in a tared dry weight tube, weighed, and filtered. The mycelium was washed by resuspension in enough distilled water to fill the tube, filtered, and the wash liquor discarded. The tube was dried to constant weight in a vacuum oven at 80° C, stoppered and capped before cooling in a desiccator, and weighed to 0.1 mg. Results were expressed as milligram of dry mycelium per gram of whole sample (mg/g WS).

When samples of less than 25 g were removed from the fermenter, somewhat high results were obtained when the dry weight was less than, and low results when it was greater than, ca. 18 mg/g WS. No explanation is offered for this effect; but when samples of greater than 25 g were taken the method was reproducible to $\pm 1.3\%$.

The Course of Accumulation of Mycelium

After a period in which little metabolic activity could be detected, mycelial accumulation started, but frequently growth was later halted for a period during which the dry weight remained constant, resulting in a stepped growth curve (Figs. 8*a*–11*a*). Subsequently growth continued until the limiting nutrient was exhausted.

FIG. 8. The course of a glucose-limited fermentation.
(*a*) One scale unit ≡ 1 pH unit ○-----○; 5.0 mg/g WS dry weight ●———●; and utilization of 20 mmoles/l. total inorganic N ▲———▲; 100 mmoles/l. glucose □———□; 2.5 mmoles/l. P ×———×; 1.0 mmole/l. Mg +———+; and 1.0 mmole/l. K △———△.
(*b*) One scale unit ≡ 5.0 g/100 g dry mycelium, fat ×———×, carbohydrate +———+; 2.0 mg/g WS, fat -----, carbohydrate —— ——.
(*c*) One scale unit ≡ 50 μg P/g WS. Total ASP □———□; 7-minute ASP ●———●; stable ASP —— ——; PL-P ■———■; PNA-P + DNA-P + MP △———△; PNA-P ×———×; DNA-P +———+; and MP -----.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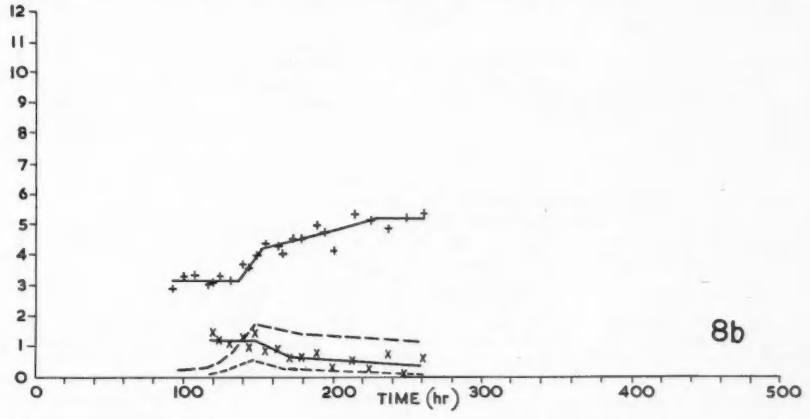
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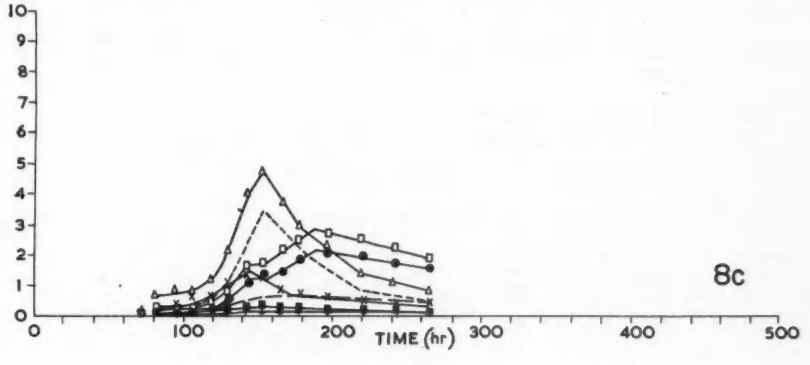

8c
TIME (hr)

FIG. 9. The course of a nitrogen-limited fermentation.

(*a*) One scale unit = 1 pH unit ○-----○; 5.0 mg/g WS dry weight ●———●; and utilization of 20 mmoles/l. total inorganic N ▲———▲; 100 mmoles/l. glucose □———□; 2.5 mmoles/l. P ×———×; 1.0 mmole/l. Mg +———+; and 1.0 mmole/l. K △———△.

(*b*) One scale unit = 5.0 g/100 g dry mycelium, fat ×———×, carbohydrate +———+; 2.0 mg/g WS, fat -----, carbohydrate ——·——; 20 iodine ▲———▲, acid ○———○, and saponification ●———● values.

(*c*) One scale unit = 50 μg P/g WS. Total ASP □———□; 7-minute ASP ●———●; stable ASP ——·——; PL-P ■———■; PNA-P + DNA-P + MP △———△; PNA-P ×———×; DNA-P +———+; and MP -----.

FIG. 10. The course of a phosphate-limited fermentation (PmNG).

(*a*) One scale unit = 1 pH unit ○-----○; 5.0 mg/g WS dry weight ●———●; and utilization of 20 mmoles/l. total inorganic N ▲———▲; 100 mmoles/l. glucose □———□; 2.5 mmoles/l. P ×———×; 1.0 mmole/l. Mg +———+; and 1.0 mmole/l. K △———△.

(*b*) One scale unit = 5.0 g/100 g dry mycelium, fat ×———×, carbohydrate +———+; 2.0 mg/g WS, fat -----, carbohydrate ——·——; 20 iodine ▲———▲, acid ○———○, and saponification ●———● values.

(*c*) One scale unit = 50 μg P/g WS. Total ASP □———□; 7-minute ASP ●———●; stable ASP ——·——; PL-P ■———■; PNA-P + DNA-P + MP △———△; PNA-P ×———×; DNA-P +———+; and MP -----.

FIG. 11. The course of a magnesium-limited fermentation (MNPG).

(*a*) One scale unit = 1 pH unit ○-----○; 5.0 mg/g WS dry weight ●———●; and utilization of 20 mmoles/l. total inorganic N ▲———▲; 100 mmoles/l. glucose □———□; 2.5 mmoles/l. P ×———×; 1.0 mmole/l. Mg +———+; and 1.0 mmole/l. K △———△.

(*b*) One scale unit = 5.0 g/100 g dry mycelium, fat ×———×, carbohydrate +———+; 2.0 mg/g WS, fat -----, carbohydrate ——·——; 20 iodine ▲———▲, acid ○———○, and saponification ●———● values.

(*c*) One scale unit = 50 μg P/g WS. Total ASP □———□; 7-minute ASP ●———●; stable ASP ——·——; PL-P ■———■; PNA-P + DNA-P + MP △———△; PNA-P ×———×; DNA-P +———+; and MP -----.

FIG. 12. The course of a phosphate-limited fermentation (PmNG).

(*a*) One scale unit = 1 pH unit ○-----○; 5.0 mg/g WS dry weight ●———●; and utilization of 20 mmoles/l. total inorganic N ▲———▲; 100 mmoles/l. glucose □———□; 2.5 mmoles/l. P ×———×; 1.0 mmole/l. Mg +———+; and 1.0 mmole/l. K △———△.

(*b*) One scale unit = 5.0 g/100 g dry mycelium, fat ×———×, carbohydrate +———+; 2.0 mg/g WS, fat -----, carbohydrate ——·——; 20 iodine ▲———▲, acid ○———○, and saponification ●———● values.

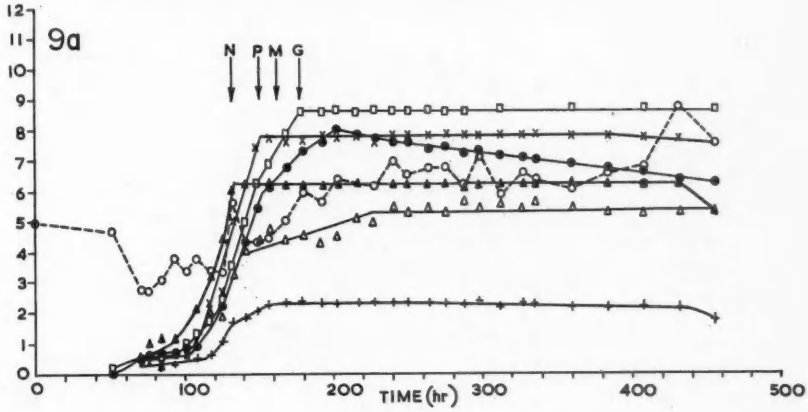
(*c*) One scale unit = 10 μg P/g WS. Total ASP □———□; 7-minute ASP ●———●; stable ASP ——·——; PL-P ■———■; PNA-P + DNA-P + MP △———△; PNA-P ×———×; DNA-P +———+; and MP -----.

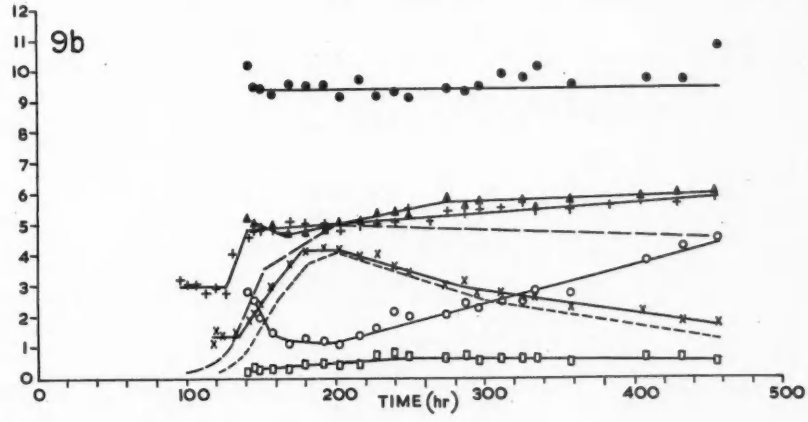
FIG. 13. The course of a magnesium-limited fermentation (MNGp).

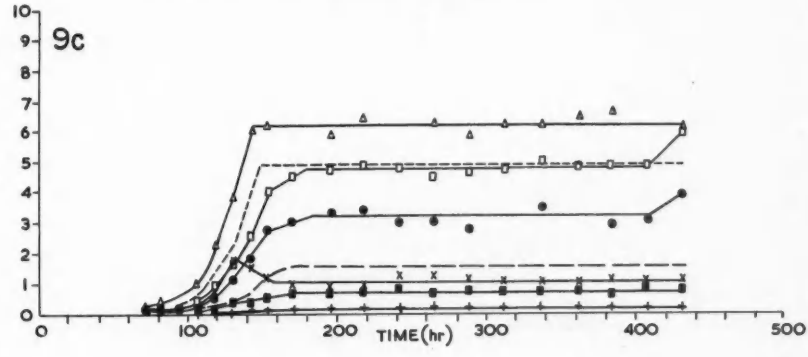
(*a*) One scale unit = 1 pH unit ○-----○; 5.0 mg/g WS dry weight ●———●; and utilization of 20 mmoles/l. total inorganic N ▲———▲; 100 mmoles/l. glucose □———□; 2.5 mmoles/l. P ×———×; 1.0 mmole/l. Mg +———+; and 1.0 mmole/l. K △———△.

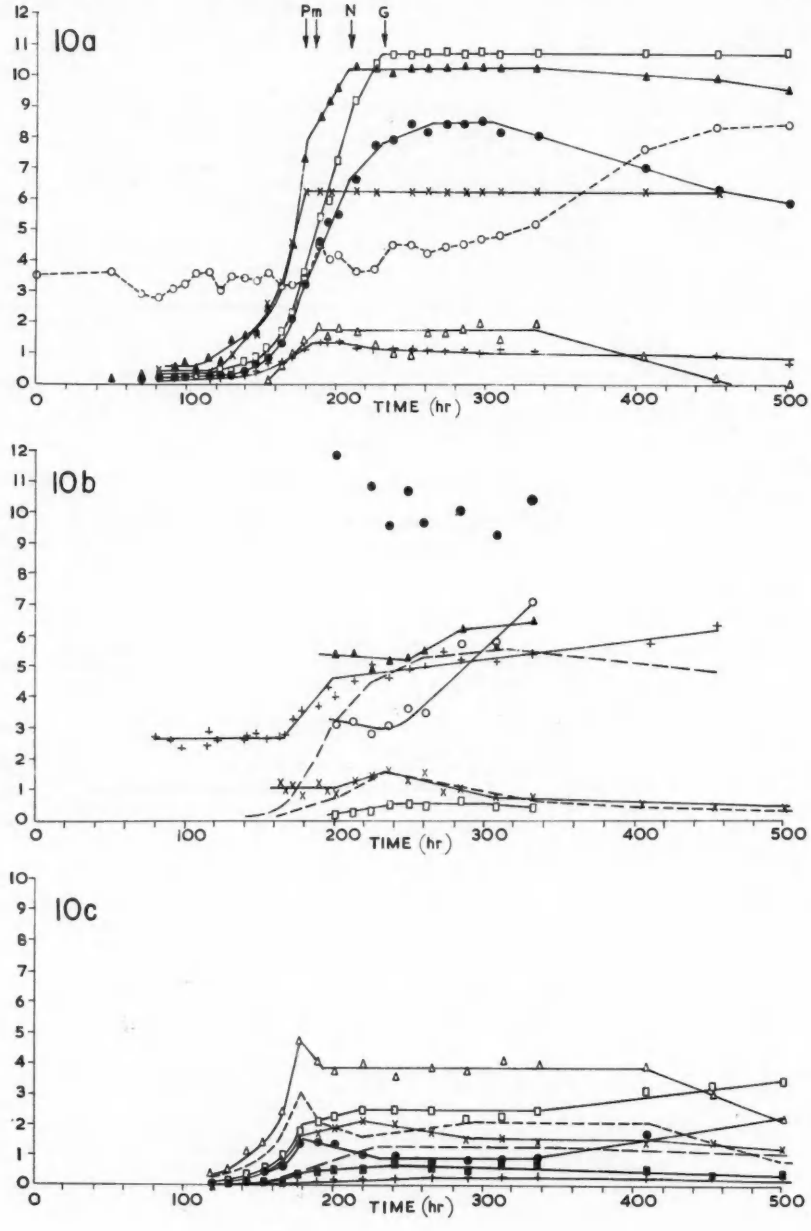
(*b*) One scale unit = 5.0 g/100 g dry mycelium, fat ×———×, carbohydrate +———+; 2.0 mg/g WS, fat -----, carbohydrate ——·——; 20 iodine ▲———▲, acid ○———○, and saponification ●———● values.

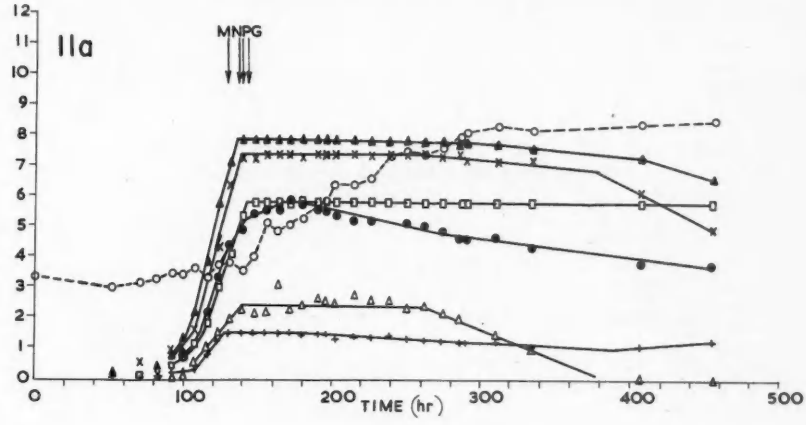
(*c*) One scale unit = 50 μg P/g WS. Total ASP □———□; 7-minute ASP ●———●; stable ASP ——·——; PL-P ■———■; PNA-P + DNA-P + MP △———△; PNA-P ×———×; DNA-P +———+; and MP -----.

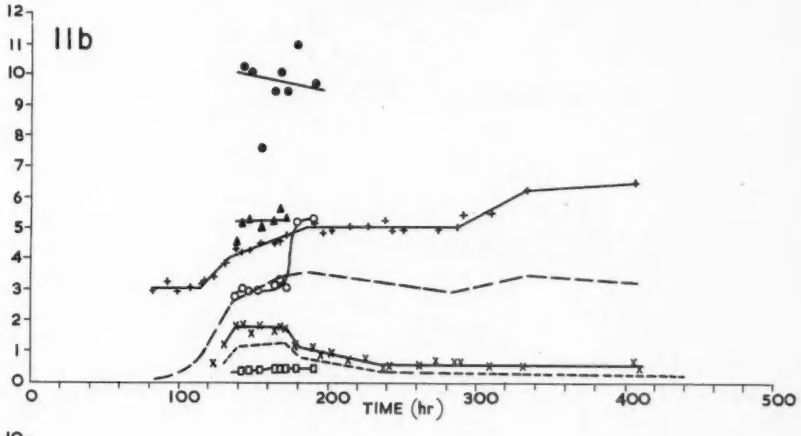

9a
N
P
M
G
TIME (hr)

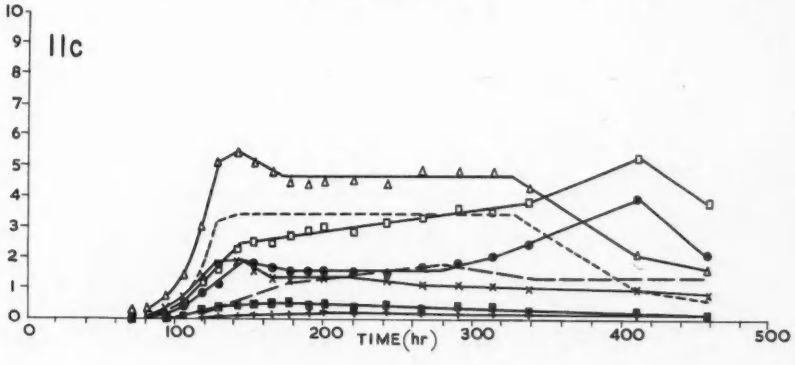

9b
TIME (hr)

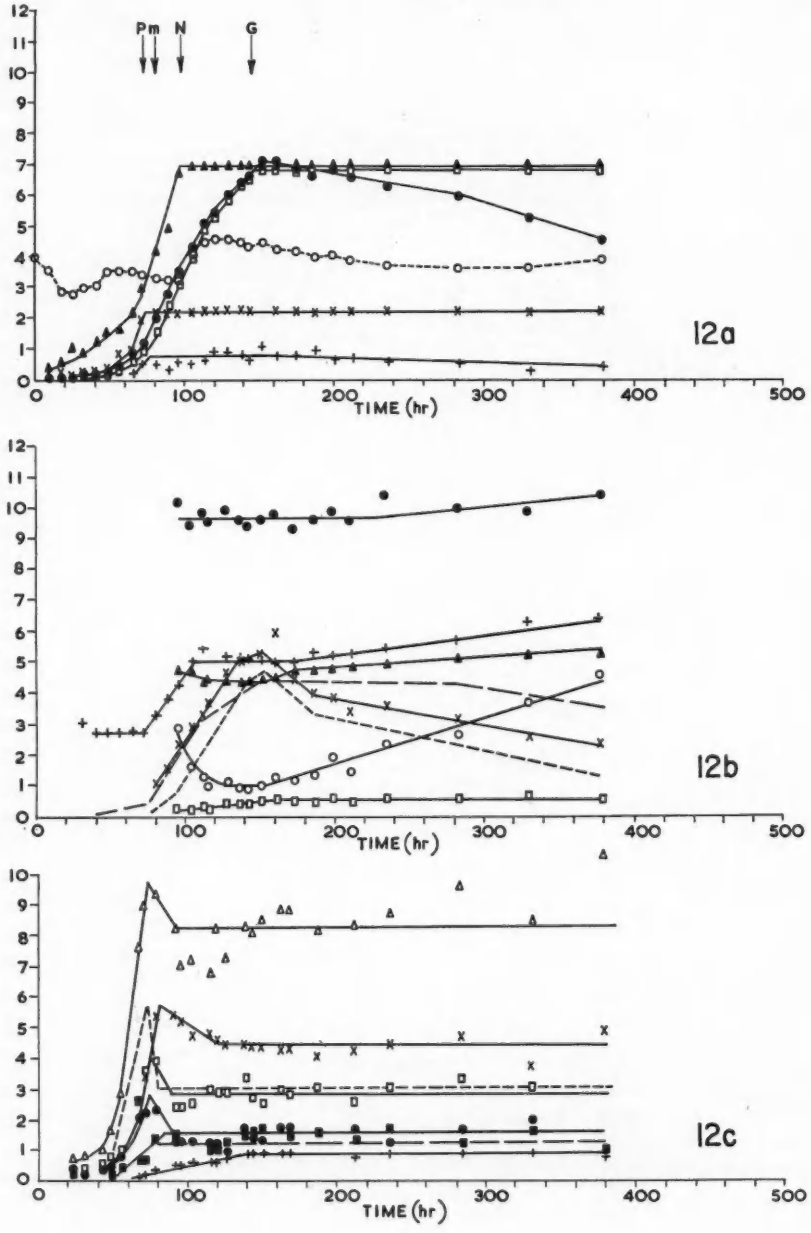

9c
TIME (hr)

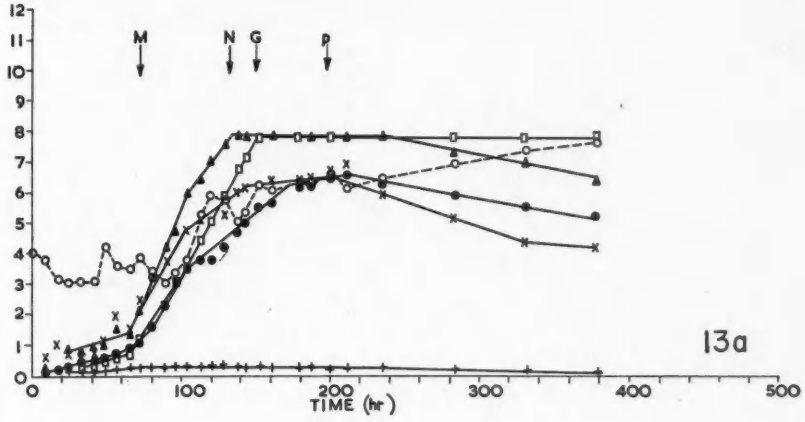

10a
Pm
N
G
TIME (hr)
10b
TIME (hr)
10c
TIME (hr)

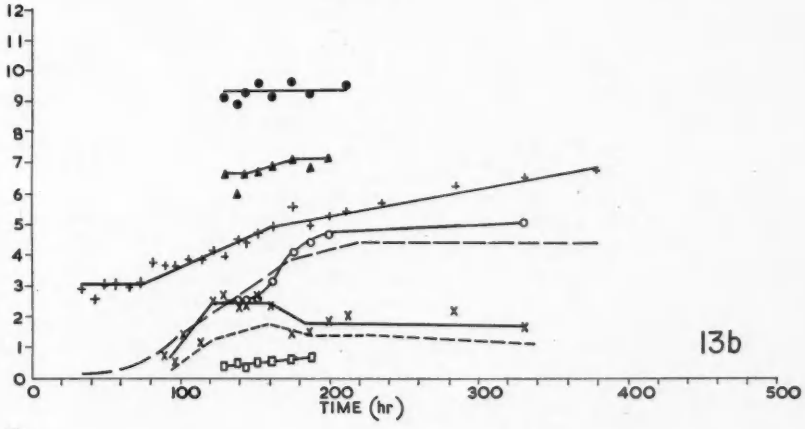

IIa
MNPG
TIME (hr)

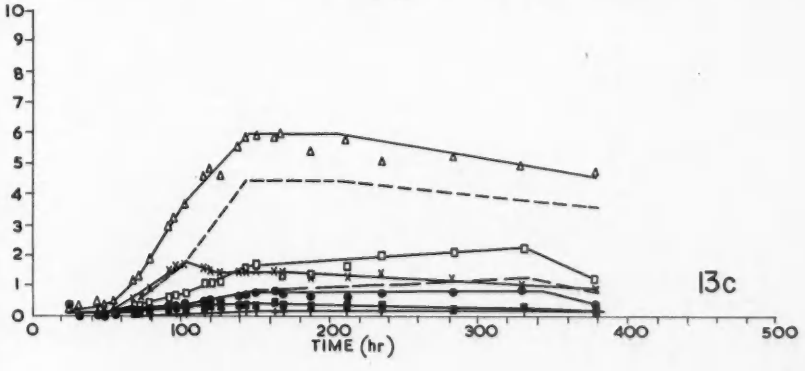

IIb
TIME (hr)


IIc
TIME (hr)


Pm
N
G
TIME (hr)
12a
TIME (hr)
12b
TIME (hr)
12c


M
N
G
P
13a
TIME (hr)


13b
TIME (hr)


13c
TIME (hr)

In G-limited media (Fig. 8*a*), only small increases in dry weight occurred after the time of glucose exhaustion, and the dry weight began to decrease rapidly within 20 hours.

When either nitrogen (Fig. 9*a*), phosphate (Fig. 10*a*), or magnesium (Fig. 11*a*) was the limiting nutrient, the rate of accumulation of dry weight frequently decreased when the limiting nutrient was exhausted. Further such decreases were often noted when other nutrients were subsequently exhausted. When only small excesses of glucose remained at the time when the limiting nutrient was exhausted, the dry weight reached its maximum value soon after the time when glucose was exhausted, irrespective of the sequence in which other nutrients were exhausted, and then decreased slowly for the remainder of fermentation. In N-limited fermentations, in which large glucose excesses remained after the exhaustion of nitrogen, the dry weight reached a maximum value, of approximately twice that at the time of nitrogen exhaustion, at which it remained constant until the excess glucose was exhausted. Thereafter it decreased for the remainder of fermentation.

Morphology of Mycelium

The photomicrographs of fresh mycelium (Plate I) were recorded on Kodak 'Microfile' film, using a ×45, 0.95 N.A. fluorite oil-immersion objective. In all fermentations, the hyphae were sparsely branched early in growth. As growth continued, branching increased and the length of the hyphae decreased. The cytoplasm was usually seen to contain a few small granules in each cell, together with one or two small vacuoles which later increased in size. Occasional cells, singly or in groups, contained very finely granular cytoplasm in which it was difficult to discern vacuoles or the type of granule noted above. Both types of cell were present throughout the period prior to the exhaustion of the first nutrient, but the former type was always predominant (Fig. 14). The mycelium was pale salmon pink. Changes in morphology after the exhaustion of the first nutrient depended upon which nutrient limited.

Soon after the exhaustion of glucose in G-limited fermentations the hyphae began to break down into shorter units, and vacuolation increased rapidly giving rise to cells with the cytoplasm only lining the inside of the cell wall, or occasionally with two large vacuoles within the cell. Later, many cells broke down entirely losing their contents to the medium (Fig. 15). The mycelium became yellow grey, the medium becoming a pale, slightly opalescent yellow.

After the exhaustion of nitrogen in N-limited fermentations, oil globules could be seen in the cells (Fig. 16), increasing in size and number until glucose was in turn exhausted. Their nature was confirmed in that they took up 'Sudan' stains, and were removed by ether, but not by acid, during the fat extraction process. These globules were invariably visible when the mycelium

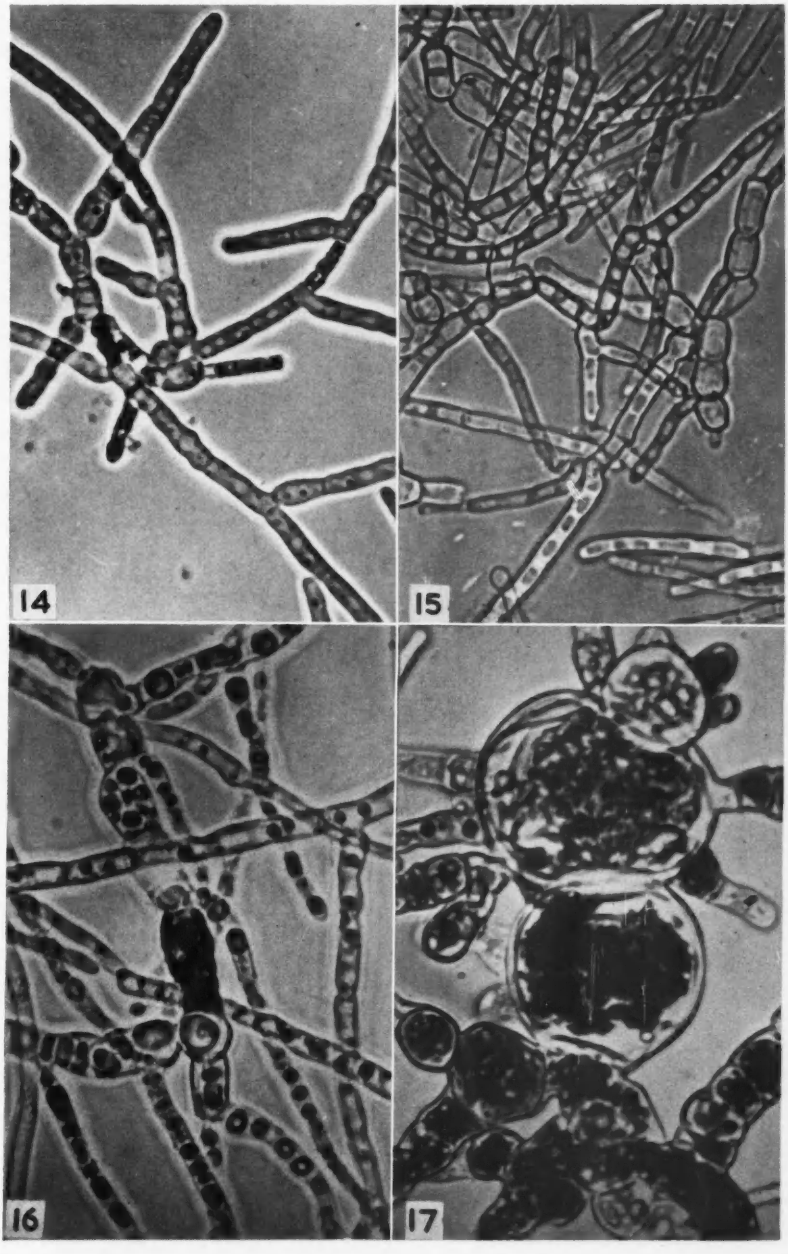
---

FIG. 14. Mycelium of *G. fujikuroi* in the presence of all nutrients (balanced phase). (×1000)

FIG. 15. Mycelium of *G. fujikuroi* after exhaustion of glucose in glucose-limited fermentation (terminal phase). (×1000)

FIG. 16. Mycelium of *G. fujikuroi* after exhaustion of nitrogen in nitrogen-limited fermentation (maintenance phase). (×1000)

FIG. 17. Abnormal globose cells of *G. fujikuroi*. (×880)


14
15
16
17

contained more than 10% of fat. When only few oil globules were produced they could be seen to decrease in size and number later in fermentation, but when many globules were produced they coalesced later in fermentation and no visual estimate of the changing oil content was possible. The presence of many globules precluded observations on the vacuolation of the cells, but by the time they had decreased, vacuolation had increased, although not to the same extent as in G-limited fermentations. The mycelial color intensified after the exhaustion of nitrogen, rapidly becoming purple-brown, and later purple-grey. Much of the pigment was extracted with the fat in diethyl ether; and the color was sensitive to changes in pH, being red in acid and purple in alkaline conditions. The medium was often a purple-brown color, the pigment sometimes settling out on standing in the refrigerator.

Soon after the exhaustion of nitrogen in two fermentations, groups of abnormally large globose cells appeared (Fig. 17). They contained purple-brown granules, oil globules, and granules. These cells, which were presumably those of *G. fujikuroi*, persisted throughout the remainder of fermentation, their contents behaving in the same way as those of normal cells.

After the exhaustion of phosphate in P-limited fermentations the mycelium continued to resemble that present before the exhaustion of any nutrient, until such time as either glucose or nitrogen was in turn exhausted. The subsequent changes in morphology resembled those in either G- or N-limited fermentations.

After the exhaustion of magnesium in M-limited fermentations, oil globules became visible while nitrogen was still being used. These became increasingly abundant until the time when glucose was exhausted, and thereafter they decreased.

Changes in pH

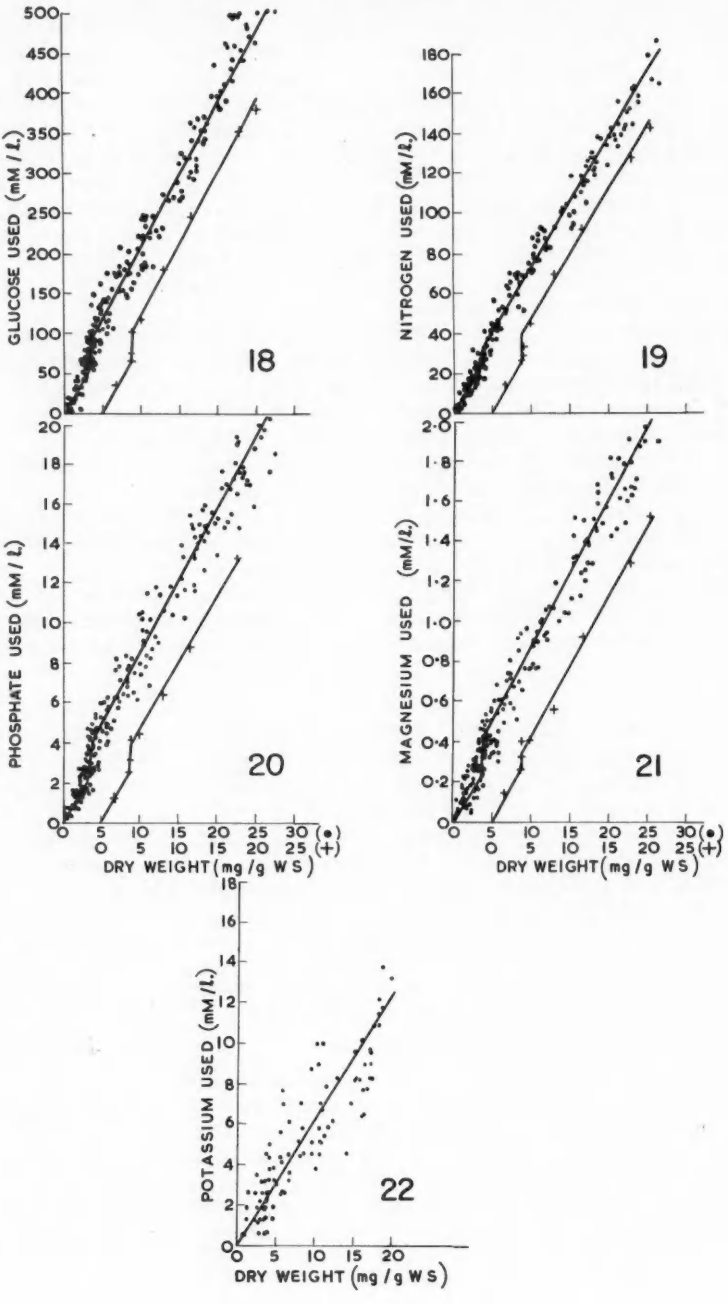
After remaining approximately constant early in fermentation, the pH decreased, reaching a value of ca. 3.0–2.8 at the time when the dry weight 'step' began. It increased slowly throughout the period of the 'step', and continued to increase during the period of rapid growth. Later changes in pH depended largely upon the sequence in which glucose and nitrogen were exhausted, irrespective of the time when phosphate or magnesium was exhausted. When glucose was exhausted before nitrogen (Fig. 8*a*) the pH continued to increase rapidly to values greater than pH 8, where it remained throughout the terminal period of mycelial breakdown. When nitrogen was exhausted before glucose (Figs. 9*a*, 10*a*, 11*a*) the continued increase was less rapid, and was interrupted by a period of constancy when large excesses of glucose were present.

The Uptake of Nutrients

Methods

Glucose was originally measured gravimetrically (1) but later an adaptation of a more rapid colorimetric method (3) was introduced.

Total inorganic N (NH_3-N + NO_3-N) was measured by the method of Conway (13). Samples were analyzed in the presence and absence of Devarda's alloy. Nitrite was assumed to be negligible and NO_3-N was calculated by difference. NH_3-N was also sometimes measured after steam distillation (33).


GLUCOSE USED (mM/L)
18
NITROGEN USED (mM/L)
19
PHOSPHATE USED (mM/L)
20
MAGNESIUM USED (mM/L)
21
DRY WEIGHT (mg/g WS) (•)
DRY WEIGHT (mg/g WS) (+)
POTASSIUM USED (mM/L)
22
DRY WEIGHT (mg/g WS)

Phosphate was measured by the method of Berenblum and Chain (5).

Magnesium was measured by the method of Gasser (21) but the buffer of Harvey, Komarmy, and Wyatt (22) was used and the range restricted to 0–25 μg magnesium.

Potassium was measured on an EEL flame photometer.

The Course of Uptake of Nutrients

The uptake of glucose, total inorganic N, phosphate, magnesium, and potassium began when mycelium began to accumulate, and continued until the time when the limiting nutrient was exhausted. NH_3-N was not taken up during the dry weight 'step', but the uptake of NO_3-N continued. Glucose, phosphate, magnesium, and potassium also continued to be taken up during the 'step', but at greatly reduced rates. During the period of rapid growth, both NH_3-N and NO_3-N were taken up, but NH_3-N was used at a greater rate, and was therefore always exhausted ca. 4 hours before the exhaustion of NO_3-N. To minimize confusion, only total inorganic N has been plotted in Figs. 8*a*–13*a*.

When phosphate was the first nutrient to be exhausted, the uptakes of magnesium and potassium ceased at, or shortly after, this time (Figs. 10*a*, 12*a*). Decreases in the rate of uptake of the residual nutrients were frequently noted at the time when the limiting nutrient was exhausted, and at the time of exhaustion of subsequent nutrients. In experiment 3 (Figs. 12*a*, 13*a*), similar decreases were noted after the exhaustion of the limiting nutrient and before that of the second external nutrient. In the P-limited fermentation this decrease occurred when internal reserves of metaphosphate ceased to be used. It is possible that the similar decrease in the M-limited fermentation indicated that reserves of magnesium were exhausted at this time.

Despite these decreases in rate, the uptake of each residual nutrient continued after the exhaustion of the limiting nutrient until such time as it was exhausted, or the dry weight reached a maximum value. In many fermentations this value was reached at the time when glucose was exhausted; but in some fermentations in experiment 1 the dry weight reached a constant maximum value in the presence of a large excess of glucose, and in these conditions the uptake of all residual nutrients except glucose ceased at the time this value was reached. Glucose, however, continued to be metabolized, in some cases for as long as 1000 hours.

Nutrient Uptake in Relation to Dry Weight

In Figs. 18–21 the uptake of glucose, inorganic N, phosphate, and magnesium have been plotted in relation to dry weight up to the time of glucose exhaustion, irrespective of the sequence in which the nutrients were exhausted. The data are massed from all fermentations in experiment 2, which was designed so that the limiting nutrient would be exhausted at a dry weight of 20 mg/g WS. Data from single fermentations are also shown.

These figures show that the uptake of each nutrient increased with increasing dry weight until a dry weight of ca. 3 mg/g WS was reached and dry weight accumulation ceased for a period. All nutrients continued to be taken up during

FIGS. 18–22. Nutrient uptake in relation to dry weight. Data massed from experiment 2 ●; data from single fermentation +.

this period. After the dry weight 'step' the uptake of all nutrients was linearly proportional to the dry weight until the time of glucose exhaustion. Despite the frequent decreases in the rate of both dry weight accumulation and of nutrient uptake after the exhaustion of the limiting nutrient, and the changing composition of the mycelium, the proportionality between dry weight and nutrient uptake remained linear at dry weights greater than 20 mg/g WS.

Subsequent to the dry weight 'step', therefore, the formation of 1 mg dry mycelium/g WS was accompanied by the uptake of 18.0 mmoles/l. glucose, 6.6 mmoles/l. nitrogen, 0.74 mmole/l. phosphate, and 0.075 mmole/l. magnesium. The analyses for potassium were not as accurate as the other measurements, due to the large dilutions necessary to bring the concentration within the range of the method used, but the data in Fig. 22 suggest that there was a similar relationship between dry weight and potassium uptake until the exhaustion of the limiting nutrient, and that the formation of 1 mg dry mycelium/g WS was accompanied by the uptake of ca. 0.6 mmole/l. potassium. These values are equivalent to the uptake of 3.24 mg glucose, 0.092 mg nitrogen, 0.023 mg phosphorus, 0.0018 mg magnesium, and 0.023 mg potassium during the formation of 1 mg dry mycelium.

These values, which are analogous to economic coefficients, confirm those obtained from experiment 1, which were used to calculate the composition of the media used in experiment 2. The fact that in this experiment either glucose, nitrogen, phosphate, or magnesium was exhausted as planned at a dry weight of 20 mg/g WS, and that the remaining nutrients were subsequently exhausted in selected sequences, is a further confirmation of such proportionality.

Liberation of Components to the Medium

During the breakdown of the mycelium at the end of fermentation, in all conditions ammonia, phosphate, magnesium, and potassium were returned to the medium. The apparent uptake of magnesium late in fermentation, after much had been returned to the medium, cannot be explained, but it is improbable that it represents utilization by the mold.

Changes in Mycelial Fat

Methods

The extraction method was based on that of Smedley-MacLean (47). Either a weighed sample from the fermenter was taken into a dry weight tube, filtered, and washed as already described, or a known weight of dry mycelium was used. The mycelium was then treated for 60 minutes at 100° C with 15 ml 1 *N* HCl, filtered, washed until free of acid, and dried *in vacuo* at 80° C before extracting for 42 hours with diethyl ether. The solvent was removed and the fat was weighed. The method was reproducible to 2.3% on a sample of mycelium containing 21.7% fat. Only 47% of the fat was extracted from this sample when the acid treatment was omitted.

Not enough fat (100–350 mg) was obtained from normal samples for standard analyses on separate aliquots (9). The analyses were therefore adapted and made consecutively on each small sample. After being weighed, the fat in the extraction flask was dissolved in hot ethanol (neutralized to phenolphthalein with 0.1 *N* alcoholic KOH) and then titrated with aqueous 0.1 *N* KOH, again using phenolphthalein. The acid value was calculated from this titration. The

same sample was then saponified by refluxing for 1 hour with a measured excess of alcoholic KOH, and the residual KOH was titrated with 0.1 *N* HCl against phenolphthalein. The value calculated from this titration is a measure of the esterified acids, and the saponification value was calculated by adding this to the acid value. Most of the alcohol was then distilled from the sample. The fatty acids were liberated by the addition of *N* H_2SO_4 and extracted into diethyl either, washed free of acid, and the solvent removed *in vacuo*. The iodine value of these fats was measured (9) after they had been weighed. The phospholipid content of the fat was measured among the phosphorus-containing fractions. The free fatty acid content of the fat was calculated by expressing the acid value as a percentage of the saponification value. This agreed with conventionally made measurements.

The results from the micromethod agreed with those from the conventional macromethods. Using samples of linseed oil they gave, respectively, acid values of 5.1 and 5.2, saponification values of 188 and 189, iodine values of 180 and 183, and free fatty acid contents of 2.7 and 2.7%. Similar agreement was obtained with mycelial fat.

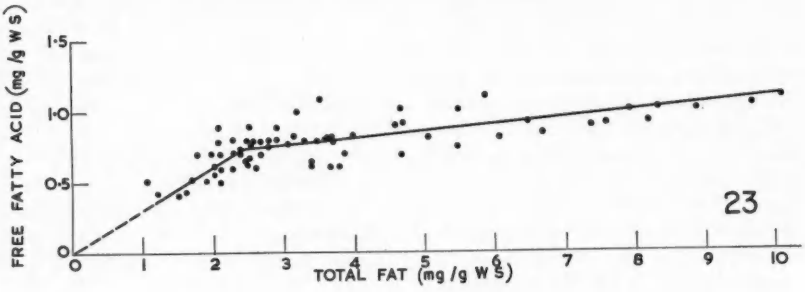
The Course of Accumulation of Fat

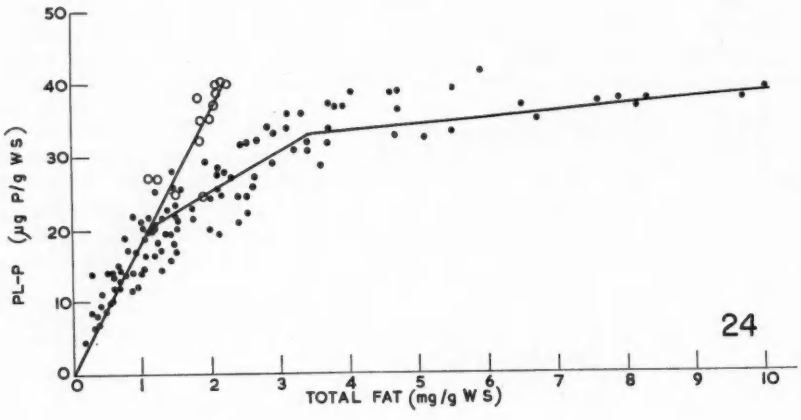
Mycelial fat (mg/g WS) accumulated until the exhaustion of the limiting nutrient. Thereafter no further increases occurred in G-limited fermentations (Fig. 8*b*); but fat continued to accumulate, sometimes at increased rates, in N-, P-, and M-limited fermentations until the time when the dry weight reached its maximum value, after which fat decreased, at first rapidly, and then at a greatly reduced rate (Figs. 9*b*–11*b*).

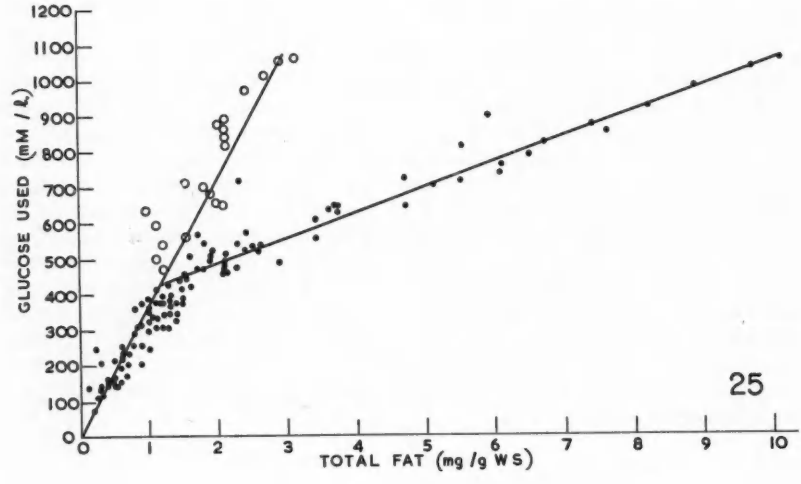
The fat content of the mycelium (% dry mycelium) remained constant at a value of ca. 6% until the exhaustion of the limiting nutrient in all fermentations. No increase was subsequently found in G-limited fermentations (Fig. 8*b*). In P-limited fermentations it remained constant at ca. 6% until the exhaustion of either glucose or nitrogen, whichever was sooner, and subsequent increases were found only when nitrogen was exhausted before glucose (Fig. 10*b*). In the fermentation in experiment 3 in which phosphate was exhausted at a relatively low dry weight (Fig. 12*b*), the fat content of the mycelium increased after the internal reserves of metaphosphate ceased to be used, although neither glucose nor nitrogen had been exhausted. Increases were also initiated by the exhaustion of nitrogen in N-limited fermentations (Fig. 9*b*), and of magnesium in M-limited fermentations (Figs. 11*b*, 13*b*). All increases continued until the time when the dry weight reached its maximum value, except in the fermentation limited by magnesium at a relatively low dry weight (Fig. 13*b*), in which it reached a constant value before nitrogen was exhausted.

In N-limited fermentations in which the dry weight reached its maximum potential value in the presence of a large excess of glucose, as in some fermentations in experiment 1, not only the dry weight, but also the fat, remained constant until the glucose was in turn exhausted. Under these conditions the dry mycelium contained ca. 45% of fat, irrespective of the mycelial concentration.

In all conditions the fat content of the mycelium decreased after the exhaustion of glucose.


FREE FATTY ACID (mg /g W S)
1·5
1·0
0·5
0
0 1 2 3 4 5 6 7 8 9 10
TOTAL FAT (mg /g W S)
23


PL-P (µg P/g W S)
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
TOTAL FAT (mg/g W S)
24


GLUCOSE USED (mM / L)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
TOTAL FAT (mg /g W S)
25

Acid, Iodine, and Saponification Values

In those fermentations in which fat in excess of 6% dry mycelium was formed (Figs. 9*b*–13*b*), the acid and iodine values of the fat decreased during the period in which the fat content of the mycelium increased, and they increased during the period of fat utilization, thereafter remaining constant or increasing slightly during the terminal phase. The saponification value of the fat remained constant or decreased slightly during each fermentation. In all conditions it remained within the range 180–230.

Free Fatty Acid

Free fatty acid (mg/g WS) increased until shortly after the time when the fat reached its maximum value, and thereafter remained constant or decreased slightly for the remainder of fermentation (Figs. 9*b*–13*b*).

In Fig. 23, massed data for free fatty acid from experiment 2 have been plotted in relation to total fat during the period of fat accumulation. The 20 mg dry mycelium/g WS present at the time of exhaustion of the first nutrient contained ca. 1.2 mg fat/g WS. No data for free fatty acid were obtained until after this value for fat was reached. The data in Fig. 23 show that thereafter free fatty acid increased with increasing fat until a value of 2.4 mg fat/g WS was reached, and suggest that throughout this period the fat contained ca. 30% free fatty acid. Thereafter varied, and sometimes large, increases in fat (from 2.4–10 mg/g WS) were accompanied by only small increases in free fatty acid (from 0.7–1.1 mg/g WS). Similarly, varied and sometimes large decreases in the fat later in fermentation were accompanied by only slight changes in the amount of free fatty acid.

Phospholipid

Phospholipid (expressed as μg P/g WS) increased until the time when the fat content of the mycelium began to increase, and then increased at a reduced rate until the time when the fat had reached its maximum value. Thereafter it remained constant or decreased slightly for the remainder of the fermentation.

In Fig. 24 massed data for phospholipid-P from experiment 2 have been plotted in relation to fat up to the time when the fat reached its maximum value. Values at dry weights greater than 20 mg/g WS in P-limited fermentations have been distinguished. The phospholipid-P content of the fat was constant at a value of ca. 1.8% until a value of 1.2 mg fat/g WS (contained in 20 mg dry mycelium/g WS) was reached. This constancy was maintained after the exhaustion of phosphate in P-limited fermentations until the time when nitrogen or glucose, whichever was sooner, was exhausted. After the exhaustion of the limiting nutrient in N- and M-limited fermentations, the phospholipid content of the fat decreased, until finally varied, and sometimes large, increases

FIG. 23. The relation between free fatty acid and total fat. Data massed from experiment 2.

FIG. 24. The relation between phospholipid phosphorus and total fat. Data massed from experiment 2. ○ denotes values from phosphorus-limited fermentations at dry weights greater than 20 mg/g WS.

FIG. 25. The relation between glucose uptake and the accumulation of fat. Data massed from experiment 2. ○ denotes values from phosphorus-limited fermentations at dry weights greater than 20 mg/g WS.

in fat (from 3.4–10 mg/g WS) were accompanied by only small increases in phospholipid (from 33–39 μg P/g WS).

Changes in the Composition of the Fat

Before nutrient limitation the fat, as analyzed from substantial samples from 80-l. scale fermentations, was of approximately constant composition (Table II), and qualitatively was characterized by relatively high iodine and acid values and by a high free fatty acid content. The phospholipid phosphorus content of 18 μg PL-P/mg dry mycelium (Fig. 24) is equivalent to a phospholipid content (expressed as lecithin) of 45% of the fat and accounts for the measured (41%) combined fatty acid content of the fat (Table III). These combined fatty acids, like the free fatty acids (which comprised 39% of the fat) were relatively unsaturated. The unsaponifiable material comprised ca. 9% of the fat and was characterized by a bright yellow color, a high iodine value, and the presence of carotenoids was indicated by the Carr–Price reaction. The presence of ergosterol (59) was confirmed by its infrared spectrum after crystallization from the unsaponifiable fraction. In general, therefore, during the period prior to the exhaustion of the limiting nutrient the small constant amount of fat which was accumulated in the mycelium contained ca. 39% free fatty acid, ca. 45% phospholipid, and ca. 9% unsaponifiable material.

After the exhaustion of phosphate in the P-limited fermentations in experiment 2, the composition of the fat remained virtually unchanged until after the exhaustion of nitrogen in the presence of glucose. In the remainder of this section the changes in the composition of the fat after the exhaustion of nitrogen in N- and P-limited fermentations, and after the exhaustion of magnesium in M-limited fermentations in experiment 2, will be described.

During the periods in which the fat content of the mycelium increased, the acid and iodine values decreased, while free fatty acid and phospholipid only increased slightly. This implies that the fat formed in this period included only small amounts of the more unsaturated fatty acid and phospholipid, and consisted largely of relatively saturated combined fatty acids. This was confirmed by the macroanalyses shown in Table III (columns 2 and 3), in which it will be seen that the combined fatty acid increased to over 80% of fat (section B), and that the proportion of saturated acid increased (section C). The increase in the combined fatty acid fraction cannot be accounted for by phospholipid, and therefore this increase must be due to the accumulation of more saturated triglycerides.

Conversely, throughout the period of rapid fat utilization, decreases in fat were accompanied by increases in acid and iodine values, and by only slight changes in free fatty acid and phospholipid, implying that the more saturated triglycerides formed previously were being utilized preferentially. As a result, the more intimately bound free fatty acid, phospholipid, and unsaponifiable material made progressively increasing contributions to the total fat; and when finally the fat content of the mycelium reached low values, the fat again consisted largely of free fatty acids, phospholipid, and unsaponifiable material, as in the period before nutrient limitation.

The Accumulation of Fat in Relation to Glucose Utilization

It has been shown above that, in conditions where only a small excess of

TABLE II

Analysis of fat from varied mycelial concentrations before the exhaustion of any nutrient

Dry wt. mycelium, mg/g WS	Acid value	Saponification value	Iodine value	Free fatty acid % of fat	Fat, mg/g (calc.)	Free fatty acid	
						mg/g WS	% of fat-free dry mycelium
1.1	104	191	136	54.5	0.066	0.036	3.5
1.3	95	183	131	51.9	0.078	0.040	3.3
1.5	117	209	136	56.0	0.090	0.050	3.5
2.3	104	192	143	54.2	0.138	0.075	3.5
2.9	90	189	141	47.6	0.174	0.083	3.0
3.2	87	191	145	45.5	0.192	0.087	2.9
6.5	105	199	127	52.8	0.390	0.206	3.4
7.1	94	178	149	52.8	0.426	0.225	3.4
8.3	87	179	141	48.6	0.498	0.242	3.1
9.1	80	180	139	44.4	0.546	0.242	2.8
12.0	63	192	120	32.8	0.720	0.236	2.1
17.6	113	206	117	54.9	1.056	0.580	3.5

TABLE III

Detailed confirmatory analyses of fat at different stages of growth from large samples and with conventional analyses (9)

Column No.		1	2	3
Harvest relative to nitrogen exhaustion		Before	After	After
III(A). Whole fat				
% dry mycelium		6	16	35
Acid value		62	31	12
Iodine value		125	91	96
Saponification value		189	196	198
III(B). Fractionation data				
Free fatty acids	: % total fat	39	17	8
	: Iodine value	130	94	101
Combined fatty acids	: % total fat	41	70	82
	: Iodine value	113	90	94
	: Mean molecular weight	283	280	279
Unsaponifiable material	: % total fat	8.7	4.5	2.9
	: Iodine value	173	214	167
III(C). Separation data*				
Solid acids	: % total fat	20	26	28
(saturated)	: Iodine value	44†	4	8
	: Mean molecular weight	277	264	276
	: Melting point (°C)	—	50–53	51–53
Liquid acids	: % total fat	61	56	61
(unsaturated)	: Iodine value	146	125	134
	: Mean molecular weight	291	282	281
Unsaponifiable material	: % total fat	8.7	4.7	3.1
	: Iodine value	172	210	—

*Twitchell (53).
†This high value results from the high proportion of unsaturated acids which made separation of uncontaminated solid acids difficult.

glucose remained after the exhaustion of the limiting nutrient, the dry weight and the fat reached maximum values at the time when glucose was exhausted. This suggested that, except when the maximum of 45% fat was reached in the presence of excess glucose, the amount of fat formed was determined by the amount of glucose available. In Fig. 25, massed values for fat from experiment 2 are plotted in relation to the utilization of glucose up to the time when glucose was exhausted or, for P-limited fermentations, when nitrogen was exhausted if this occurred before glucose exhaustion. The data from P-limited fermentations have been distinguished from those from N- and M-limited fermentations. 1.2 mg fat/g WS would be present when the limiting nutrient was exhausted at a dry weight of 20 mg/g WS. It will be seen that there is a direct relation between fat formation and glucose uptake up to 1.2 mg fat/g WS, and that this relation remained unchanged after the exhaustion of phosphate in P-limited fermentations. In N- and M-limited fermentations, however, more fat was formed in relation to the glucose used after the exhaustion of nitrogen or magnesium, and this relation is linear. It can therefore be calculated that in this latter period the uptake of 1 g glucose was accompanied by the production of 78 mg fat, corresponding to a fat coefficient of 7.8, compared with 1.9 before the exhaustion of the limiting nutrient.

Mycelial Carbohydrate

Methods

Total mycelial carbohydrate was usually measured on the dry mycelium remaining after dry weight measurement. This was ground, sieved (100 mesh), and weighed into centrifuge tubes in which it was soaked with water, spun, and the supernatant retained. The residue was wet milled with a metal pestle and pooled with the supernatant. Aliquots of this suspension were taken for measurement. Fresh mycelium was filtered, washed, and similarly milled before measurements were made.

One milliliter of mycelial suspension, diluted to contain 10–60 μg of carbohydrate was pipetted into a tube and immersed in cold water. Five milliliters of 0.1% anthrone in 87.5% (v/v) H_2SO_4 was run in rapidly and swirled to mix while still cold. The tube was loosely stoppered and placed for exactly 6 minutes in a boiling water bath and then rapidly cooled. The absorption was read at 590 mμ. Results were expressed as the glucose equivalent. Chitin, the presence of which was indicated in extracted mycelium (54), gave a reaction equivalent to 1% that of the same weight of glucose. Similar results were obtained with glucosamine.

Fractionation of Mycelial Carbohydrate

Two fractions, one soluble in hot water, and one in hot dilute HCl, were clearly defined. The residual mycelium could not be further fractionated. The water-soluble fraction was removed by two 30-minute treatments with 15 ml of water at 100° C in the dry weight tube assembly. The acid-soluble fraction was then removed from the same sample by three 30-minute extractions with 0.5 *N* HCl at 100° C. Neither stronger acid, nor alkali in various concentrations, gave reproducible measurements. The residual mycelium was then wet milled and prepared in the same way as whole mycelium. The total carbo-

hydrate measured in these three fractions agreed with the total carbohydrate measured on the whole mycelium.

The Course of Accumulation of Carbohydrate

Mycelial carbohydrate (mg/g WS) accumulated until the time when the dry weight reached its maximum value, and thereafter remained constant, or decreased slightly for the remainder of fermentation (Figs. 8*b*–13*b*).

The carbohydrate content of the mycelium (% dry mycelium) remained constant in all conditions at a value of ca. 16% until the first nutrient was exhausted. In P- and M-limited fermentations it began to increase at the time when the limiting nutrient was exhausted (Figs. 10*b*–13*b*), and in N-limited fermentations it began to increase when NH_3-N was exhausted, before the subsequent exhaustion of NO_3-N. In these three types of fermentation, therefore, the carbohydrate content of the mycelium increased while nitrogen was still being taken up, and this increase continued until either glucose was in turn exhausted, or a value of ca. 32% was reached. Values greater than 32% were sometimes observed late in fermentation, due to the liberation of other cell components.

Changes in Mycelial Carbohydrate

The fractionation of the mycelial carbohydrate was undertaken to determine whether it was possible to distinguish between the carbohydrate laid down as a constant proportion of the mycelium in the presence of all nutrients, and that laid down later.

Mean values from several fermentations in experiment 1 showed that while the carbohydrate remained constant at ca. 16% dry mycelium, it consisted of 8.8% water-soluble, 62.5% acid-soluble, and 28.8% residual carbohydrate. The increase in carbohydrate later in fermentation could be largely accounted for as acid-soluble carbohydrate. The water-soluble fraction remained constant as a percentage of fat-free mycelium, while the residual fraction increased from 4.3% to a maximum value of 8% fat-free mycelium.

The water-soluble carbohydrate contained 10% pentose (7), and 10% free reducing sugar (17). The acid-soluble carbohydrate contained 3% pentose and 20% free reducing sugar, but the latter probably arose by hydrolysis during extraction. The residual fraction, which contained negligible pentose, could only be extracted by concentrated acid, and was obtained in solution only as reducing sugar. All carbohydrate, except free reducing sugar, was ethanol precipitable in alkaline conditions. The separated alcohol precipitates, from both water- and acid-soluble fractions, could be accounted for as reducing sugar and pentose after hydrolysis.

Paper chromatograms were run on Whatman No. 4 paper in butanol:acetic acid:water (39), with extracts before and after hydrolysis, neutralized when necessary with "Amberlite" IR4B. For this purpose the residual fraction was dissolved in hot 5 *N* HCl, and was therefore already hydrolyzed. Four types of spot were observed: I, on the starting line; II, with the same R_f as glucose; III, with an $R_{glucose}$ of 1.3; and IV, a pentose. Although spot III ran to about the same position as fructose it was probably not this sugar, since it was revealed only by aniline hydrogen oxalate, and not by naphthoresorcinol

(39). The development of these spots was similar throughout fermentation, and showed that mycelial carbohydrate was largely present as polymers, not as simple sugars, and suggested that all fractions had a common basic unit, which was probably, but not definitely, glucose.

The fractions defined were not therefore clearly relatable to the phase of growth.

Fat- and Carbohydrate-free Dry Weight

With a mycelial organism, such as *G. fujikuroi*, no measurement of cell population can be made. Although fat- and carbohydrate-free dry weight must include chitin and possibly other uncharacterized compounds, we regard it as the best criterion for the amount of living matter available from the results. Periods in which the fat- and carbohydrate-free dry weight increased are regarded as periods of cell proliferation.

The Course of Accumulation of Fat- and Carbohydrate-free Dry Weight

In Figs. 26–29, the course of accumulation of dry weight, fat-free dry weight, and fat- and carbohydrate-free dry weight are shown for typical G-, N-, P-, and M-limited fermentations.

When glucose was the first nutrient to be exhausted (Fig. 26), the fat- and carbohydrate-free dry weight reached its maximum value at the time of glucose exhaustion, and began to decrease rapidly within 20 hours.

When nitrogen was the first nutrient to be exhausted (Fig. 27), the rate of increase of fat- and carbohydrate-free dry weight decreased considerably at the time of nitrogen exhaustion, only small amounts were subsequently formed, and it soon reached a maximum value at which it remained approximately constant until after the exhaustion of glucose. During this period the dry weight reached a maximum value and either immediately decreased (in fermentations with small excesses of glucose) or remained constant and then decreased (in fermentations with large excesses of glucose). These changes were almost entirely accounted for by changes in fat. When, subsequently, the fat- and carbohydrate-free dry weights also began to decrease, the rate was considerably less rapid than in G-limited fermentations.

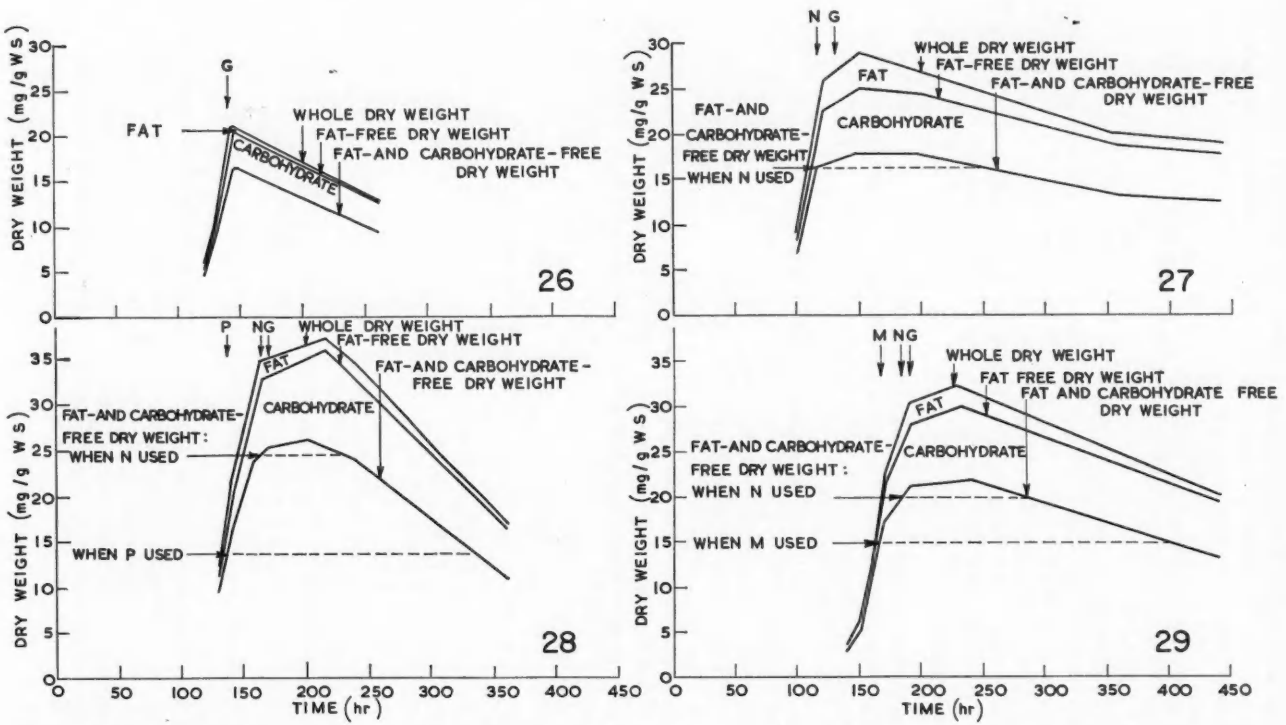
When phosphate or magnesium was the first nutrient to be exhausted (Figs. 28, 29), the fat- and carbohydrate-free dry weight continued to increase until the time when either glucose or nitrogen, whichever was sooner, was in turn exhausted; and thereafter the pattern of events resembled those in either G- or N-limited fermentations.

These results show that proliferation was terminated by the exhaustion of either glucose or nitrogen, but not by that of phosphate or magnesium.

Phosphorus-containing Fractions of the Mycelium

Methods

The fractionation of the phosphorus-containing compounds was developed to measure acid-soluble phosphorus (ASP), phospholipid phosphorus (PL-P), pentosenucleic acid phosphorus (PNA-P), and desoxypentosenucleic acid phosphorus (DNA-P). In addition to these, it was found that the mycelium of *G. fujikuroi* contained two metaphosphate fractions, one of which (7-minute



FIGS. 26–29. Changes in whole, fat-free, and fat- and carbohydrate-free dry weight during glucose- (Fig. 26), nitrogen- (Fig. 27), phosphorus- (Fig. 28), and magnesium- (Fig. 29) limited fermentations.

ASP) was soluble in cold trichloroacetic acid (TCA), while the other (MP) was extracted with the nucleic acids. The presence of metaphosphate in both extracts was confirmed by the metachromatic test and by the characteristic gelatinous precipitate obtained with barium (55).

The whole fractionation was made on the same weighed sample, of greater than 25 g, while it remained in the same dry weight tube, extracts being collected in standard flasks.

The filtered mycelium was washed with ice-cold water and then extracted at 1° C, a 3-ml rapid wash followed by a 15-ml extraction for 30 minutes both with 10% TCA, and then a 10-ml wash with ice-cold water. The TCA extracts and final water wash were made to 50 ml and measurements were made of total ASP (after perchloric acid digestion) and of 7-minute ASP (after hydrolysis for 7 minutes at 100° C in *N* HCl).

After traces of TCA had been discarded with a further water wash at 1° C, the mycelium was suspended in 5 ml ice-cold water and 15 ml cold ethanol added with stirring. After 10 minutes at 1° C the liquid was collected by filtration, the residue extracted for 10 minutes with cold ethanol, and again filtered before being extracted overnight with boiling diethyl ether. All three extracts were pooled and aliquots were evaporated to dryness, digested with perchloric acid, and the phosphate content was measured (PL-P).

The residue was freed from traces of ether in a vacuum oven at 80° C, and was then extracted with 15 ml 0.5 *N* perchloric acid for 30 minutes at 70° C. The extract was cooled immediately after filtration, and two further extractions were made from the residue into a separate receiver, with the same reagent at 70° C. The three extracts were pooled and made to 50 ml with 0.5 *N* perchloric acid.

Analyses were made on the extract for total P after perchloric acid digestion, for DNA-P by the diphenylamine method of Burton (10) using a standard of DNA of known P content which had been treated in the same way as the samples, and total NA-P by the absorption at 260 mμ using a standard of PNA of known P content similarly treated. PNA-P and MP were calculated from these measurements by difference.

Twelve replicate samples were removed from a fermentation and fractionated by the above procedure. The mean values (μg P/g WS) obtained were, total ASP 112.2±4.4, 7-minute ASP 84.1±4.3, stable ASP 28.0±2.8, PL-P 14.2±1.8, total NA-P+MP 182.5±5.2, total NA-P 29.0±0.4, DNA-P 4.47±0.15, PNA-P 24.5±0.4, and MP 153.6±5.3.

Measurements of native ortho-P in the ASP fraction were not made. Values for 7-minute ASP therefore represent native ortho-P, acid-soluble metaphosphate, and labile organic phosphorus; but since the metaphosphate was the largest component, changes in 7-minute ASP probably reflect changes in this compound. Pyrophosphate could not be precipitated from the ASP fraction with cadmium ions nor with zinc ions (25).

Most of the lipid phosphorus was removed by the two ethanolic extractions, but the prolonged ether extraction also removed the mycelial fat, which might otherwise have presented a variable barrier to subsequent reagents.

PNA could not be measured in terms of its pentose moiety because *G. fujikuroi* contains other sources of pentose which were extracted with the

nucleic acids. The use of perchloric acid to remove this extract was derived from the method of Ogur and Rosen (37). Their material, however, did not contain MP; and we found that no distinct separation of PNA, DNA, and MP could be obtained using variations of temperature and perchloric acid concentration. For this reason, all were removed in one extract and distinguished as described. No measurements were made of phosphoprotein, since only a small carryover from the large MP fraction introduced considerable errors in the measurement of this small fraction. The small amount of residual phosphorus was therefore discarded.

In general, the fractionation procedure was complicated by the presence of large amounts of metaphosphate, and it is therefore not as precise as those published for animal and plant tissues (37, 45, 56). Similar difficulties have been reported when working with yeast by Juni *et al.* (25). The values obtained, however, reveal the main aspects of phosphorus metabolism throughout the course of fermentation.

The Course of Accumulation of Phosphorus-containing Compounds

In all fermentations except that shown in Fig. 12*c*, stable ASP accumulated until the exhaustion of the limiting nutrient, and continued to increase, often at a reduced rate, for 20–40 hours thereafter. It then remained approximately constant until late in fermentation. In the fermentation shown in Fig. 12*c* it reached a constant value ca. 5 hours after the exhaustion of phosphate.

Under all conditions 7-minute ASP accumulated until the exhaustion of the limiting nutrient. In P-limited fermentations (Figs. 10*c*, 12*c*), or in fermentations in which phosphate was exhausted soon after the limiting nutrient, 7-minute ASP either remained constant, or decreased and then remained constant. In all other conditions it continued to increase for a further 15–30 hours after the exhaustion of the limiting nutrient, and then remained approximately constant for a period. In all conditions 7-minute ASP increased rapidly late in fermentation, and then decreased, this decrease being accompanied by a return of phosphate to the medium.

In all fermentations except that shown in Fig. 12*c*, PL-P accumulated until the exhaustion of the limiting nutrient, and then continued to increase, often at a reduced rate, until about the time when glucose was exhausted. Thereafter it decreased slowly for the remainder of fermentation, often after a period of constancy. In the fermentation shown in Fig. 12*c* it reached a constant value ca. 5 hours after the exhaustion of phosphate.

DNA-P always accumulated until the exhaustion of the limiting nutrient, and thereafter continued to increase until shortly after the time when glucose was exhausted. It then remained constant, only decreasing slowly late in fermentation.

In G- and N-limited fermentations (Figs. 8*c*, 9*c*), PNA-P accumulated until the exhaustion of the limiting nutrient, and then began to decrease, the time at which it reached its maximum value coinciding with the time of nutrient exhaustion. In G-limited fermentations this decrease, accompanied by a return of phosphate to the medium, continued until the disappearance of PNA-P. In N-limited fermentations the decrease continued for 20–60 hours, and PNA-P then remained constant during the period of fat utilization. A further decrease,

accompanied by the return of phosphate, occurred late in fermentation.

In M-limited fermentations (Fig. 11*c*), PNA-P accumulated until the time of magnesium exhaustion, and then increased slightly for about 20 hours. It then decreased, the decrease being interrupted by a period of constancy when, subsequent to magnesium exhaustion, nitrogen was exhausted before glucose. An exception to this was observed in the fermentation shown in Fig. 13*c*, in which PNA-P continued to accumulate after the exhaustion of magnesium, reaching its maximum value at a time between the exhaustion of magnesium and that of nitrogen.

In P-limited fermentations (Fig. 10*c*), PNA-P accumulated until the time of phosphate exhaustion, and thereafter continued to increase, but at a reduced rate, until such time as either nitrogen or glucose was exhausted. When glucose was exhausted before nitrogen, PNA-P decreased for the remainder of fermentation; but when nitrogen was exhausted before glucose (Fig. 10*c*), PNA-P decreased for ca. 40 hours, remained constant during the period of fat utilization, and then decreased rapidly for the remainder of fermentation. In the fermentation shown in Fig. 12*c*, PNA-P reached a maximum value ca. 5 hours after the exhaustion of phosphate, decreased for ca. 40 hours, and then remained constant.

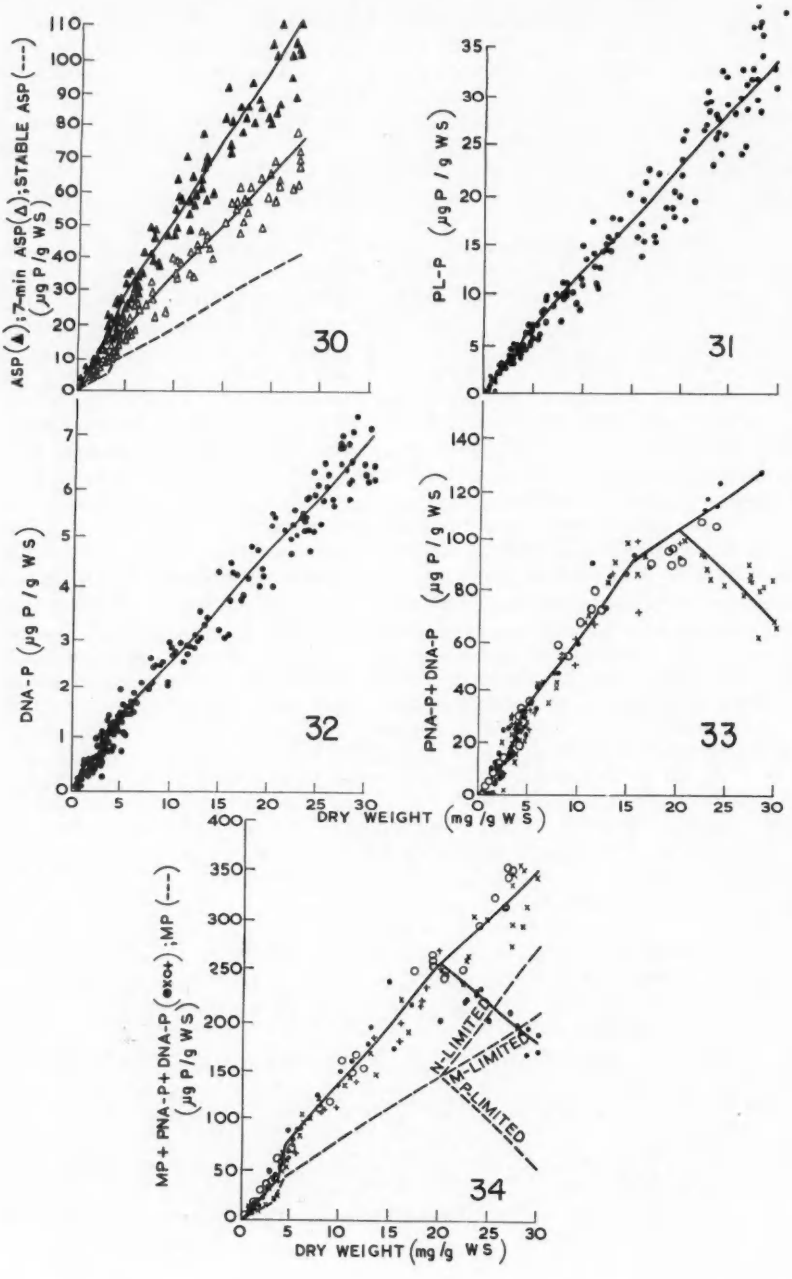
MP accumulated until the time of exhaustion of the limiting nutrient. In P-limited fermentations (Figs. 10*c*, 12*c*), it began to decrease at the time of phosphate exhaustion, and later became constant. In G-, N-, and M-limited fermentations (Figs. 8*c*, 9*c*, 11*c*, 13*c*) it continued to increase, sometimes at a reduced rate, after the exhaustion of the limiting nutrient until phosphate ceased to be taken up or was exhausted. Thereafter, in N- and M-limited fermentations it remained constant, unless phosphate was exhausted soon after the limiting nutrient when it decreased before becoming constant. In all conditions, MP decreased rapidly at the end of fermentation.

Phosphorus-containing Fractions in Relation to Dry Weight

In Figs. 30–34 the measured values from experiment 2 for phosphorus in each extract are plotted in relation to dry weight until the time of glucose exhaustion, irrespective of the sequence in which other nutrients were exhausted. Slopes representing stable ASP (Fig. 30) and MP (Fig. 34) have been calculated. The data from the four types of fermentation have been distinguished in Figs. 33 and 34.

All fractions continued to accumulate during the dry weight 'step' at ca. 3 mg dry mycelium/g WS, throughout which phosphate uptake continued. Thereafter there was a linear proportionality between dry weight and 7-minute ASP, stable ASP, PL-P, and DNA-P which continued until glucose was exhausted although the rate of dry weight production and the composition of the mycelium often varied after the exhaustion of the limiting nutrient at a dry weight of 20 mg/g WS.

Since DNA-P was linearly proportional to dry weight until glucose was exhausted, and contributed only ca. 4% of the total NA-P, the form of the relationship shown in Fig. 33 can be attributed to PNA-P. The proportionality between dry weight and the accumulation of PNA-P did not extend beyond a dry weight of 15 mg/g WS, and at greater dry weights PNA-P contributed


ASP(▲);7-min ASP(△);STABLE ASP(---) (μg P/g WS)
30
PL-P (μg P / g WS)
31
DNA-P (μg P / g WS)
32
PNA-P+DNA-P (μg P / g WS)
33
DRY WEIGHT (mg/g WS)
MP+PNA-P+DNA-P (●x○+) ;MP (---) (μg P/g WS)
N-LIMITED
M-LIMITED
P-LIMITED
34
DRY WEIGHT (mg/g WS)

less to the mycelium. After the exhaustion of nitrogen in N-limited fermentations, PNA-P decreased with increasing dry weight. The decrease was linear in each fermentation. Similar decreases occurred in P-limited fermentations after nitrogen exhaustion, but since the concentrations of nitrogen were varied in these fermentations such decreases began at varied dry weights, and these data have therefore been omitted from the figure.

Figure 34 shows that there was a linear proportionality between the accumulation of MP and dry weight until a dry weight of 20 mg/g WS had been reached. This relationship remained unchanged until the time of glucose exhaustion in M-limited fermentations. In N-limited fermentations more MP contributed to dry weight after the time of nitrogen exhaustion than previously. In P-limited fermentations there was a linear proportionality between the decrease in MP and the increase in dry weight after the exhaustion of phosphate.

The Distribution of Phosphorus-containing Compounds within the Mycelium

In the presence of all nutrients the contribution of phosphorus-containing fractions to dry weight remained constant until a dry weight of 20 mg/g WS was reached, except in the case of PNA-P in which the contribution decreased to 2.9 μg PNA-P/mg dry mycelium at a dry weight of 15 mg/g WS. The contribution of the phosphorus in the phosphorus-containing fractions to the dry mycelium at dry weights of less than 15 mg/g WS is summarized in Table IV, in which the phosphorus in each fraction is also expressed as a percentage of the total mycelial phosphorus. The approximate weight of the phosphorus-containing compounds can be calculated from their phosphorus content by the use of rather arbitrary factors. The factors used, and the contribution of each compound to dry mycelium, are also shown.

During this period the PNA-P:DNA-P ratio remained constant at a value of 24. This ratio decreased at dry weights greater than 15 mg/g WS, before the exhaustion of the limiting nutrient, due to the decreased contribution of PNA-P to dry mycelium. The two metaphosphate fractions (7-minute ASP and MP) together contributed about half the mycelial phosphorus during this period; and ca. 13% of the dry mycelium can be accounted for as phosphorus-containing compounds.

The total phosphorus found in the mycelium was equivalent to 1.77% of dry mycelium. The uptake of phosphate from the medium during the formation of 1 mg dry weight has been shown to be equivalent to a phosphorus content of 2.3% dry mycelium. The discrepancy between these values cannot be explained; but it may be pertinent that one value is derived from measurements made on culture filtrate, and the arbitrary assumption of a density of 1 for both mycelium and culture filtrate, while the other is derived from direct measurements.

After the exhaustion of phosphate in P-limited fermentations the decrease of MP suggested that phosphorus in the MP fraction contributed to increases

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FIGS. 30–34. The accumulation of phosphorus-containing compounds in relation to dry weight. Data massed from experiment 2. In Figs. 33 and 34, values from glucose- +, nitrogen- ×, phosphorus- ●, and magnesium- ○ limited fermentations.

TABLE IV

The distribution of the phosphorus-containing compounds in the mycelium at dry weights of less than 15 mg/g WS

	Phosphorus		Reference compound		
Fraction	% dry mycelium	% total P	Name	Factor	% dry mycelium
Stable acid-soluble	0.17	9.6	Hexose phosphate	7	1.2
7-minute acid-soluble	0.30	16.9	Potassium hexametaphosphate	3.8	1.1
Phospholipid	0.106	6.0	Lecithin	26	2.7
DNA	0.022	1.2	DNA	11	0.2
PNA	0.53	29.9	PNA	11	5.8
Metaphosphate	0.64	36.2	Potassium hexametaphosphate	3.8	2.4
Total	1.77	—	—	—	13.4

in the remaining fractions. This decrease was 9.3 μg MP/g WS for every 1 mg/g WS increase in dry weight, which was more than sufficient to account for the increase of phosphorus in the remaining fractions.

The use of metaphosphate as a phosphorus reserve was confirmed by the fermentation in experiment 3 in which phosphorus was exhausted at a low mycelial concentration (Fig. 12). In this fermentation, MP began to decrease at the time of phosphate exhaustion. Before nitrogen was exhausted MP ceased to be used, and at this time the remaining phosphorus-containing compounds ceased to accumulate.

In those fermentations in which, irrespective of the limiting nutrient, nitrogen was exhausted before glucose there was a period in which the fat- and carbohydrate-free dry weight remained constant at its maximum value. During this period, all phosphorus-containing fractions remained constant within individual fermentations, stable ASP, PL-P, and DNA-P at greater values, and PNA-P at a lower value than at the time of nitrogen exhaustion. The values for 7-minute ASP and MP depended upon whether phosphate exhaustion had previously resulted in their re-use or not. It is not meaningful to compare the contribution of the phosphorus-containing fractions to whole dry weight in this period with that found early in growth, due to the varied accumulation of fat and carbohydrate; but the mean contributions to the fat- and carbohydrate-free dry weight of stable ASP, PL-P, PNA-P, and DNA-P were respectively 0.45, 0.24, 0.40, and 0.058% in this period, compared with 0.22, 0.14, 0.68, and 0.028% during early growth. The PNA-P:DNA-P ratio therefore had a value of 7, compared with 24 early in growth.

Late in all fermentations all phosphorus-containing fractions decreased, but the most rapid decreases were observed in PNA-P and in MP, the latter being accompanied by a rapid increase in 7-minute ASP before this also decreased. The MP fraction which was extracted with the NA-P fraction may consist of large metaphosphate polymers, and it is suggested that in the terminal phase these were hydrolyzed to shorter polymers which were acid-soluble and therefore measurable as 7-minute ASP. Further hydrolysis would then result in the liberation of phosphate into the medium and a decrease in 7-minute ASP, as observed.

Discussion

Fat and Carbohydrate

Until such time as one nutrient became limiting, the fat content of the dry mycelium remained constant at ca. 6%, and was of relatively constant composition. This is reminiscent of Belin's (2) fatty *élément constant*, which he regarded as an essential component of protoplasm, since it was always approximately proportional to mycelial nitrogen, and which could not decrease below a limiting value. He introduced this concept after finding that the fatty acid content of felts of *Sterigmatocystis nigra* (=*Aspergillus niger*) never decreased below a value of 1.4% of dry mycelium on prolonged starvation (59 days) on water. However, his results indicate that in this time considerable autolysis, shown by a 61% decrease in dry weight, occurred. The *élément constant* is therefore a phenomenon arising from the relative rates of breakdown of many components. The present results suggest that the nature of the fat late in

fermentation is similar to that present prior to the exhaustion of the limiting nutrient, and that the latter probably provides a more accurate measure of the proportion of intimately bound fat in living mycelium.

The exhaustion of nitrogen in the presence of glucose was shown not only to terminate cell proliferation, but also to initiate a period of fat storage. Similar results with *Rhodotorula gracilis* have been reported by Pan, Andreasen, and Kolachov (38). They found two phases, in the first of which there was a rapid increase in cell population and virtually no formation of fat; while in the second the cell population was approximately constant while fat accumulated. They did not, however, relate the time at which the change occurred to the time at which any nutrient was exhausted. Similarly, Garrido and Walker (20) grew *Aspergillus nidulans* in surface culture, and from their analyses it can be calculated that the fat-free dry weight remained approximately constant from the 4th day.

The exhaustion of nitrogen in the presence of excess glucose during fermentation is a condition analogous to that in experiments in which mycelial felts are transferred to a replacement medium containing glucose but no nitrogen. Experiments of this kind have been reported using *Aspergillus niger* (42) and *Fusarium lycopersici* (57). In both instances fat and carbohydrate were accumulated by the non-proliferating mycelium.

Like mycelial concentration, the amount and composition of the fat can be varied by the design of the fermentation medium. The amounts of free fatty acid, phospholipid, and unsaponifiable material are governed by the initial concentration of the limiting nutrient; and the amount of triglyceride subsequently accumulated in addition to these is governed by the amount of residual glucose.

We have shown that after the exhaustion of glucose in N-limited fermentations, fat decreased while the fat- and carbohydrate-free dry weight remained constant. During this period the carbohydrate did not decrease; but later, when the fat- and carbohydrate-free dry weight was decreasing, decreases in mycelial carbohydrate were found, while no anthrone-reacting material appeared in the medium.

During the development of the carbohydrate fractionation method we found that a single extraction of mycelium (from which the water-soluble carbohydrate had been removed) with either 0.5 *N*, 1 *N*, 3 *N*, or 5 *N* HCl, removed the same amount of carbohydrate; but that subsequent extractions with the more concentrated acids removed more than those with 0.5 *N* HCl. The distinction between the acid-soluble and residual fractions is therefore between carbohydrate which was readily soluble in dilute or concentrated acid and that which was only extracted by prolonged treatment with concentrated acids. This suggested that the concentrated reagents were removing a barrier from the surface of the carbohydrate, which was probably laid down in the wall membrane, and that the observed differences in extractability were the results of impregnation by other substances, some of which may be lipids. This is compatible with Thomas's (52) suggestion of a laminated wall structure.

Foster (18) has denied that fat and carbohydrate stored within the cell can properly be regarded as reserve materials. He argued that since diffusible carbon compounds which are returned to the medium, and later used, cannot

be regarded as reserve compounds, then non-diffusible compounds, such as fat, which cannot escape from the cell, also cannot be regarded as reserves. However, he did not define "reserve material". We suggest that a reserve is a substance stored within a cell which can replace the function of an external nutrient which has become unavailable. It will be shown later that this definition includes not only fat, but also acid-insoluble metaphosphate, while it excludes the carbohydrate of *G. fujikuroi*, which did not replace the function of glucose since it did not prevent mycelial breakdown, only becoming available for use by surviving cells when liberated by the breakdown of others. There is a very real ecological advantage to be derived from retaining potential carbon sources for later use within the hyphae. In natural habitats, as distinct from pure culture, materials returned to the environment may be competed for by other organisms, while those retained are reserved for the mold's use. The protection offered to the fat- and carbohydrate-free components in the presence of fat, as shown by a comparison of Figs. 26 and 27, justifies the concept that fat is functionally a reserve material.

Phosphorus-containing Compounds

We have shown that until such time as one nutrient was exhausted, the contribution of all phosphorus-containing fractions except PNA-P to the mycelium of *G. fujikuroi* remained constant, and that the PNA-P also contributed a constant proportion until the dry weight reached a value of 15 mg/g WS. In studies on the growth of yeast and bacteria in which no analyses of nutrient uptake were made, it may be inferred that no nutrient was exhausted during the period of logarithmic growth. Katchman and Fetty (26) measured the phosphorus-containing fractions throughout the growth of *Saccharomyces cerevisiae* in aerated shaken fermentation, and found that throughout the logarithmic phase the dry matter contained constant amounts of 7-minute ASP (excluding native ortho-P), labile organic ASP, stable organic ASP, PL-P, and DNA-P. Ortho-P and PNA-P, however, decreased with increasing cell count throughout the logarithmic phase. No MP was detected until the cell concentration reached 1×10^7 cells/ml, but at greater concentrations MP accumulated rapidly for the remainder of the logarithmic phase. These results are similar to those reported here, and support the view that, at least on the same type of medium, cells proliferating in nutritionally unlimited conditions are of approximately constant composition.

The occurrence of two metaphosphate fractions, of different solubility in cold TCA, has been reported for other microorganisms: for yeast (25, 26, 32), *Corynebacterium diphtheriae* (15, 41), *Aspergillus niger* (29), *Neurospora crassa* (23), and *Aerobacter aerogenes* (48). Katchman and van Wazer (27) suggested that the acid-soluble and acid-insoluble metaphosphate fractions of yeast differed in their chain lengths, and that the longer chains coprecipitated with protein in cold TCA and became insoluble, while the shorter chains were extracted. Juni *et al.* (25) studied the phosphorus-containing fractions of yeast grown in the presence of labelled phosphate during growth, and showed that the phosphorus turnover was greatest in the orthophosphate and acid-insoluble metaphosphate fractions, the last being considerably more active than the acid-soluble metaphosphate. Wiame (55) also showed that the meta-

bolic activity of the two metaphosphate fractions was different. It would seem, therefore, that the two fractions differ in metabolic function, and not merely in extractability due to differing chain lengths, a conclusion which is supported by the present results.

Metaphosphate as a Phosphorus Reserve

We have shown that when phosphate was the first nutrient to be exhausted by *G. fujikuroi*, MP acted as an internal reserve which supplied phosphorus for the continued accumulation of all other phosphorus-containing compounds. Experiments in which bacteria or yeast are transferred from a complete medium to one deficient in phosphate provide a situation analogous to the exhaustion of phosphate in submerged culture. Wiame (55) transferred metaphosphate-rich yeast to a phosphate-free medium, and found that nucleic acid synthesis was first accompanied by the disappearance of the acid-insoluble metaphosphate, and later continued at the expense of the acid-soluble metaphosphate. Similar results have been reported for *C. diphtheriae* (41) and for bakers' yeast (44).

That one role of metaphosphate is that of a phosphorus reserve therefore seems reasonably established; but such a role is not incompatible with other functions which have been suggested (26, 31, 56). Winder and Denneny (56) considered it unlikely that its role as a reserve of energy-rich bonds was significant, since they calculated that the stored energy was equivalent only to that liberated by the respiration of *Mycobacterium avium* in 1 minute. However, although the energy may not be great in terms of the whole cell, it may well be of local importance on the surfaces on which it is found (16, 31).

If metaphosphate is largely a reserve substance, it would not necessarily be expected to contribute constantly to dry weight even in unlimited conditions, nor to accumulate under all fermentation conditions. Katchman and Fetty (26) reported that the MP content of yeast cells was not constant throughout the logarithmic phase, while Duguid, Smith, and Wilkinson (14) found that the accumulation of volutin (=metaphosphate) in *Bacterium aerogenes* only occurred when the pH decreased to pH 4–5 and growth was limited. Smith, Wilkinson, and Duguid (48) grew *A. aerogenes* on media deficient in various nutrients. Late in fermentation they found MP in cultures limited by nitrogen or sulphur, but none in cultures limited by glucose, phosphate, or potassium. They claimed that for MP accumulation, not only phosphate and an energy source are required, but also potassium and magnesium. Similarly, Sall *et al.* (41) reported that potassium enhanced the accumulation of MP in *C. diphtheriae*. Schmidt, Hecht, and Thannhauser (43) found that the uptake of ortho-P by bakers' yeast was always accompanied by the absorption of cations in amounts sufficient to neutralize the ortho-P and MP which accumulated in the cells. They also found that, in potassium-deficient media, phosphate uptake was slow and soon ceased, while the absence of magnesium sulphate only reduced the rate slightly. Our own results with *G. fujikuroi*, in conditions in which neither sulphate nor potassium was ever exhausted, are similar to these results. When phosphate was the first nutrient to be exhausted, not only the accumulation of MP, but also the uptakes of magnesium and potassium soon ceased. When magnesium was the first nutrient to be exhausted, the uptake

of both potassium and phosphate, and the accumulation of MP continued. In N-limited fermentations the accumulation of MP and the uptake of phosphate, magnesium, and potassium continued during the period of fat storage. In G-limited fermentations, MP accumulation and nutrient uptake ceased.

The Effect of Exhaustion of Nutrients

When any nutrients were exhausted, decreases in the rate of mycelial accumulation, and of the rate of uptake of the remaining nutrients, were frequently observed. Apart from this general effect, the results of the exhaustion of any nutrient depended upon its position in the sequence of nutrient exhaustion.

The Exhaustion of Glucose

In the absence of a carbon source in the medium or as mycelial carbon reserve, there were decreases in fat- and carbohydrate-free dry weight, mycelial breakdown and the return of components to the medium. The effect of glucose exhaustion therefore depended upon whether or not the mold had accumulated mycelial reserves.

When glucose was the limiting nutrient, the dry mycelium contained only ca. 6% of fat, and breakdown began within 20 hours of the exhaustion of glucose. Other nutrients continued to be taken up, but such uptake also ceased within 20 hours of glucose exhaustion.

When glucose was exhausted before nitrogen in P- and M-limited fermentations, a pattern similar to that in G-limited fermentations was found, except that the short period of continued nutrient uptake was sometimes slightly extended.

When glucose was exhausted after nitrogen in N- and P-limited fermentations, and after magnesium in M-limited fermentations, the dry weight always reached its maximum value after the time of glucose exhaustion, but although there was sometimes a time interval as long as 20 hours between these events, the increase in dry weight after the exhaustion of glucose was always small, and the rate of increase, low. This increase may result from the utilization of non-reducing carbon compounds in the medium which were formed earlier in growth. The time when the dry weight reached its maximum value approximately coincided with the time when fat, carbohydrate, free fatty acid, DNA-P, and PL-P reached maximum values; and the acid and iodine values of the fat reached minimum values.

Glucose was the only nutrient so far studied which never ceased to be taken up while any remained in the medium.

The Exhaustion of Nitrogen

Nitrogen is known to be distributed in the mold between protein, PNA, DNA, PL, chitin, and their unpolymerized components, and other nitrogenous compounds may be present. The absence of a nitrogen source would be expected to prevent continued proliferation.

In general, after the exhaustion of nitrogen there were only small increases in the fat- and carbohydrate-free dry weight, which reached a constant value soon after this time.

In N-limited fermentations, NH_3-N was always exhausted before NO_3-N,

and the carbohydrate content of the mycelium began to increase at the time when NH_3-N was exhausted. At the time when NO_3-N was in turn exhausted, or when nitrogen was the first nutrient to be exhausted after phosphate in P-limited fermentations, PNA-P reached its maximum value, and the fat content of the mycelium began to increase.

Nitrogen exhaustion subsequent to glucose exhaustion in G- or P-limited fermentations had little effect, because the absence of a carbon source had already initiated breakdown.

The exhaustion of nitrogen in the presence of glucose, after magnesium exhaustion in M-limited fermentations, had little effect on the PNA-P, fat, or carbohydrate content of the mycelium, since the exhaustion of magnesium had already affected these in the way characteristic of nitrogen exhaustion.

The Exhaustion of Phosphate

Phosphorus is known to occur in the mycelium in stable ASP, PL-P, PNA-P, and DNA-P and, in addition to these, two metaphosphate fractions, 7-minute ASP and MP, the latter of which could be drawn upon as a phosphorus reserve in the absence of an external phosphorus source. The exhaustion of phosphate from the medium, therefore, did not result in large changes in metabolism while such reserves were available.

When phosphate was the first nutrient to be exhausted the uptakes of magnesium and potassium ceased almost at once. Thereafter the phosphorus of the MP fraction supported the continued proliferation of the mold, while the carbohydrate, but not the fat, content of the mycelium began to increase.

When phosphate was exhausted soon after nitrogen in N-limited fermentations, the MP reserves were drawn upon in the same way as in P-limited fermentations.

The exhaustion of phosphate in G- and M-limited fermentations had little effect on the course of such fermentations.

One fermentation (Fig. 12) was designed so that phosphate would be exhausted at a relatively low mycelial concentration in the presence of excesses of other nutrients large enough to allow the reserves of MP to be exhausted before any other external nutrient. The carbohydrate content of the mycelium began to increase at the time when phosphorus was exhausted from the medium. Five hours later, at the time when the internal MP ceased to be used, all other phosphorus-containing fractions ceased to accumulate, but the carbohydrate content of the mycelium continued to increase, while the fat content began to increase, and the rates of mycelial accumulation and nitrogen uptake decreased. Fat- and carbohydrate-free dry weight continued to accumulate, but at a slightly reduced rate, until nitrogen was in turn exhausted.

The Exhaustion of Magnesium

When magnesium was the first nutrient to be exhausted, PNA-P virtually ceased to accumulate, but the fat- and carbohydrate-free dry weight continued to increase, and the fat and carbohydrate contents of the mycelium began to increase. The exhaustion of magnesium in G- or N-limited fermentations had little effect on the course of such fermentations. In P-limited fermentations, magnesium uptake ceased shortly after the time of phosphate exhaustion, and

so magnesium was never exhausted in such fermentations.

One fermentation (Fig. 13) was designed so that magnesium would be exhausted at a relatively low mycelial concentration in the presence of large excesses of all other nutrients, so that the period between the exhaustion of magnesium and that of the subsequent nutrient would be extended. It was found that during this period the PNA-P reached a maximum value, and that at this time there were decreases in the rate of mycelial accumulation and of phosphate and nitrogen uptake. The fat- and carbohydrate-free dry weight continued to increase until the subsequent exhaustion of nitrogen. This complex of events is very similar to that occurring at the time when internal reserves of MP ceased to be used, and it is suggested that at this time some uncharacterized internal reserve of magnesium was exhausted.

The Phases of Growth

Apart from convenience in describing the course of fermentation, the definition of growth phases is only justified if they correspond to different types of metabolism during the life of the mold. Here the varied results are co-ordinated into a pattern which shows that such growth phases are related to the changing nutritional status of the mold.

The Balanced Phase

The balanced phase is defined as the period of rapid growth and nutrient uptake which extended from the onset of growth until such time as the first nutrient was exhausted. As stated above, this period could be interrupted for a period by one or more dry weight 'steps' at the lower mycelial concentrations.

During the balanced phase the accumulation of 1 mg dry mycelium was accompanied by the uptake of 3.24 mg glucose, 0.093 mg nitrogen, 0.023 mg phosphorus, 0.0018 mg magnesium, and ca. 0.02 mg potassium. The mycelium contained 0.17% stable ASP, 0.30% 7-minute ASP, 0.106% PL-P, 0.022% DNA-P, 0.53% PNA-P, 0.64% MP, 6% fat, and 16% carbohydrate. The fat contained ca. 39% free fatty acid, ca. 45% phospholipid, and ca. 9% unsaponifiable material, and was characterized by relatively high acid and iodine values. The carbohydrate consisted of 8.8% water-soluble, 62.5% acid-soluble, and 28.8% residual carbohydrate. All these relations were maintained until the exhaustion of the limiting nutrient, except that less PNA-P contributed to the mycelium at dry weights of greater than 15 mg/g WS, and that in N-limited fermentations the carbohydrate content of the mycelium began to increase in the presence of NO_3-N after the exhaustion of NH_3-N.

In this phase, fat, carbohydrate, and the phosphorus-containing compounds contributed 6, 16, and 13%, respectively, to the dry mycelium. More recent work indicates that the mycelium in this phase contains ca. 40% protein and less than 5% chitin, leaving ca. 20% uncharacterized.

In general, both the composition and appearance of the mycelium and its nutritional requirements remained remarkably constant throughout the balanced phase, independent of varied initial nutrient concentrations. We regard this phase as a period of rapid proliferation in which an apparently preferred constant form of mycelium was formed in nutritionally unlimited conditions.

The Transition Phase

In M-limited fermentations, the fat and carbohydrate contents of the mycelium began to increase at the time when magnesium was exhausted from the medium, while the uptake of nutrients, including nitrogen, continued accompanied by substantial increases in fat- and carbohydrate-free dry weight. These increases continued until nitrogen or glucose was exhausted, after which proliferation virtually ceased.

Similarly, in P-limited fermentations, the uptake of nutrients, and substantial increases in fat- and carbohydrate-free dry weight continued after the exhaustion of phosphate from the medium. The carbohydrate content of the mycelium increased and, in the fermentation in which the utilization of MP ceased, while nitrogen was still being taken up, the fat content of the mycelium also increased. These increases continued until nitrogen or glucose was in turn exhausted. It has been shown that each 1 mg dry mycelium of this continued growth was supported by a decrease of 9.3 μg MP.

Similarly, in N-limited fermentations, there was a short period between the exhaustion of NH_3-N and that of NO_3-N in which the mold continued to proliferate, while the carbohydrate content of the mycelium increased.

These periods have the common characteristic that the mold continued to proliferate, but that the composition of the mycelium was changing from that in the balanced phase. The transition phase may therefore be defined as the period between the time of exhaustion of the first nutrient and that of nitrogen or glucose, whichever was sooner. Those nutrients which continued to be taken up during this phase contributed the same proportion to the dry mycelium as throughout the balanced phase, although the rates of both growth and nutrient uptake were frequently reduced.

It is probable that there are several types of transition phase, depending upon the nature of the limiting nutrient. No decrease in the rate of mycelial accumulation was detected when NH_3-N was exhausted in the presence of NO_3-N but such decreases occurred when phosphate or magnesium was exhausted, suggesting that although continued proliferation in these fermentations was supported by internal reserves of phosphorus, and possibly of magnesium, such reserves were less readily available than the external nutrient.

In the two fermentations in experiment 3 in which phosphate or magnesium was exhausted at a relatively low mycelial concentration in the presence of large excesses of nitrogen and glucose, it was noted that in the period between the exhaustion of the limiting nutrient and the subsequent exhaustion of nitrogen, PNA-P reached a maximum value, after which the fat- and carbohydrate-free dry weight continued to increase, and nitrogen continued to be taken up. This change in metabolism occurred at the time when the MP reserves ceased to be used in the phosphate-limited fermentation, and may have occurred when a reserve of magnesium was exhausted in the magnesium-limited fermentation. These results therefore suggest another kind of transitional metabolism, in which protein, but not PNA-P, accumulation continues.

The Storage Phase

The storage phase started at the time when proliferation ceased as a result of nitrogen exhaustion in the presence of excess glucose, and the fat- and car-

bohydrate-free dry weight reached a maximum value. During this phase, phosphate and glucose continued to be taken up when present in the medium, as also did magnesium and potassium, except in P-limited fermentations. All contributed the same proportion to dry mycelium as earlier in growth, but with the exception of glucose these nutrients eventually ceased to be taken up even in the presence of an excess in the medium. Dry weight, and the fat and carbohydrate content of the mycelium increased, reaching values of 45% fat and 32% carbohydrate in the presence of sufficient glucose. The fat formed during this period, readily visible as oil globules within the cells, consisted largely of relatively saturated triglycerides, although there were also small increases in free fatty acid and phospholipid. The mycelial fat was therefore characterized by progressively decreasing acid and iodine values. Stable ASP, PL-P, and DNA-P increased to constant values; 7-minute ASP and MP also increased, except in P-limited fermentations, in which they remained constant or decreased. PNA-P decreased to a constant value. The PNA-P:DNA-P ratio therefore decreased throughout this phase.

A number of changes in metabolism were therefore initiated at the start of the storage phase. These changes all began at the same time, and continued during the storage phase, but they did not end at the same time. While, at about the time when the dry weight reached its maximum value, the uptake of nutrients other than glucose ceased; fat, carbohydrate, and free fatty acid reached maximum values; the acid and iodine values of the fat reached minimum values; and stable ASP, 7-minute ASP, PL-P, DNA-P, PNA-P, and MP all reached constant values; yet these events did not exactly coincide with one another. It is probable that a number of unrelated metabolic changes were initiated by the exhaustion of nitrogen, and each of these followed its own course towards a new equilibrium condition and ceased independently of the others. The time at which the storage phase ends is not therefore so clearly defined as that at which it starts, but for routine use the time at which the dry weight reached its maximum value is probably an adequate criterion.

The storage phase is regarded as a period during which carbon and other reserves were accumulated in cells in which the components of the living matter were being reorganized after proliferation had ceased.

The Maintenance Phase

The maintenance phase is defined as the period between the time when the dry weight reached its maximum value, and the onset of terminal breakdown.

This is a period in which few changes occurred. Fat- and carbohydrate-free dry weight, carbohydrate, free fatty acid, stable ASP, 7-minute ASP, PL-P, DNA-P, PNA-P, MP, and the pH of the medium all remained approximately constant, and the PNA-P:DNA-P ratio remained constant at a value approximately one third of that in the early balanced phase. No nutrients were taken up during this period, with the exception of glucose, in the presence of large excesses of which this phase lasted for several hundred hours. While glucose was being utilized, both fat and the whole dry weight also remained constant until glucose was exhausted, after which the decrease in dry weight could be accounted for by the decrease in the triglycerides of the fat.

The maintenance phase is regarded as a period in which external or internal carbon sources were metabolized by virtually unchanging living matter.

The Terminal Phase

The terminal phase is a period of mycelial breakdown, which began only when no utilizable carbon remained. It is characterized by increasing vacuolation and loss of cell contents; hyphal breakdown; the return of ammonia, phosphate, magnesium, and potassium to the medium; an increase in pH, often to values greater than pH 8; and a decreasing fat- and carbohydrate-free dry weight. The return of phosphate to the medium was preceded by rapid decreases in PNA-P and MP, and by increases in 7-minute ASP before it also decreased. The fat present in this phase again consisted largely of free fatty acid, phospholipid, and unsaponifiable material.

The time at which 7-minute ASP began to increase, or fat- and carbohydrate-free dry weight to decrease, might provide criteria for the time at which this phase started. However, it would not be expected that every system in the mycelium would begin to break down at exactly the same time, and for routine use the time at which components begin to return to the medium is probably an adequate criterion.

The Significance of Growth Phases

The results reported in this paper are derived from samples containing large numbers of cells, which may or may not be of similar composition. It is difficult, therefore, to assess on such evidence how closely the pattern of events found during fermentation represents the history of a single cell. Results from synchronized cultures of unicellular organisms (11, 30, 34) showed that during the log phase, DNA/cell was constant, the DNA/culture doubling at the time when the cell count doubled, while PNA and protein/culture also increased stepwise, but not coincident with the stepwise increase in cell count, or with each other. Such results emphasize that during the division cycle of a single cell its composition does not remain constant, although it may follow a constant short cycle. If this is assumed for the proliferating cells of *G. fujikuroi* it implies that the constant composition found during the balanced phase was an average value, not only from many cells but also the average of the changes throughout such a cycle. This, however, may be regarded as a refinement to, rather than a contradiction of, our concept of balanced growth.

A more serious objection to this concept arises from the fact that when the mycelial concentration exceeded 15 mg/g WS in the presence of all nutrients, the PNA in the mycelium decreased. Katchman and Fetty (26) also showed that the PNA/yeast cell was not constant throughout the log phase, but decreased with increasing cell count. Similarly, Caldwell, Mackor, and Hinshelwood (12) and Price (40) found that PNA/cell of *B. lactis aerogenes* and *Staphylococcus muscae* increased with decreasing generation time. These workers were also reporting values averaged from many cells. An explanation of such results in terms of single cells may be possible. We have shown that at the time when nitrogen was the first nutrient to be exhausted, and proliferation ceased, PNA-P/culture reached a maximum value and thereafter decreased before becoming constant. If some hyphae ceased to proliferate in the

balanced phase such changes could progressively affect the average values found. The average PNA in a large sample would then depend upon the relative rates of growth by some cells and of PNA decrease in others, and therefore the shorter the generation time the greater the average PNA/cell, as found by Caldwell *et al.* and by Price.

Much work on the effect of nutrient deficiency on microorganisms has been concerned with reducing the concentration of a nutrient in the medium, and making comparison with a control in which the medium was rich in that nutrient. The comparison is often made at an arbitrary time, when some other nutrient may have limited growth in the control. The implication in the design of such experiments seems to be that the concentration of a nutrient may affect metabolism *throughout* the course of fermentation. Although this may be true in some cases, we have shown that both the composition of the mycelium and the relative uptake of all nutrients studied remained constant within a wide range of nutrient concentrations until the time when one nutrient was exhausted. Similarly, Katchman and Fetty (26) have reported that most phosphorus-containing compounds of yeast remained constant on a cell basis throughout the log phase. Bennett and Williams (4) have shown that the growth (measured as turbidity) of four strains of bacteria was directly proportional to the accumulation of bacterial phosphorus and nitrogen. In particular, Monod (35) has shown that the growth of a number of bacteria was proportional to the amount of various sugars used, not only at the time of sugar exhaustion, but throughout the period of growth, and that the proportionality varied for one organism with different carbon sources. He predicted (36) that similar relations would obtain between growth in bacterial cultures and the uptake of other, non-carbon nutrients, and he emphasized the value of determining such ratios. He formulated the relation as

$$G = KC$$

where C represents the initial concentration of the limiting nutrient, G the 'total growth' when this has been exhausted, and K is a constant. He emphasized that the significance of 'total growth' can be interpreted only when a single known nutrient limits growth. This is confirmed by the present results, which show that the determination of K by measurement of 'total growth' could be misleading in P- and M-limited fermentations, in which 'total growth' varies with the concentration of the carbon and nitrogen sources, and not with that of the limiting nutrient. We agree that it is desirable to measure K for a wide range of nutrients and nutrient sources, and we suggest that this may be best done from measurements of growth and nutrient uptake throughout the period before the exhaustion of the limiting nutrient, since such measurements are independent of the factors controlling the final value of 'total growth'. We have presented such data in terms of nutrient uptake per milligram of dry mycelium, which is mathematically the reciprocal of Monod's constant.

The balanced phase may therefore be as general a phenomenon among microorganisms as the storage phase, and the same may be true of other phases. Published studies on the tetracycline fermentations show that these compounds are formed in conditions which resemble a transition phase. For example, Zaitseva and Orlova (61) found that oxytetracycline production of

Actinomyces rimosus occurred after external phosphorus was exhausted, while mycelial growth continued at a reduced rate, accompanied by the continued uptake of the nitrogen and carbon sources. This mycelium was morphologically distinct from that produced before the exhaustion of phosphorus.

The evidence that the phases of growth described here are not unique to *G. fujikuroi* suggests that in studies on nutrient deficiency the most useful comparison is between the metabolism of the organism before and after the exhaustion of the chosen nutrient. For the same reasons, the significance of analyses of the composition of mycelia is hard to assess unless the nutritional status at the time of harvesting is known.

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NOTES

A SOIL DILUTION PROCEDURE APPLICABLE TO RHIZOSPHERE MICROORGANISM ASSAYS[1]

L. J. HERR, C. R. WEAVER, AND R. K. HORST

The nature of plant rhizosphere soil samples (soil adhering to plant roots after removal of excess soil (3,4)) precludes accurate determination of rhizosphere soil sample weights prior to suspension of the sample in the dilution liquid. Commonly this weight of soil is estimated by various means before suspension, then accurately determined after the dilution and plating of the sample (2,4) so that microorganism counts when obtained can be presented as microorganisms per g of oven-dry rhizosphere soil. Since, in this procedure, plates are poured before the soil dilution is known, the investigator must estimate the amount of soil in the rhizosphere sample and, from the estimate, decide upon appropriate dilutions for plating. Underdilution or overdilution of given rhizosphere samples may result, depending upon the accuracy of estimation of the rhizosphere soil weights. This problem is of some significance in many types of rhizosphere investigations, and it is of particular importance when attempting direct assays of antagonistic microorganisms where the numbers of zone-producing antagonistic colonies obtained per plate must be kept within reasonable limits (1). The purpose of this paper is to describe a procedure for accurately determining rhizosphere soil sample weights prior to diluting and plating the soil.

Of necessity, such determinations must be obtained from the suspended samples. Accordingly investigations were made of the relation of weights of soil in suspension to known weights of soil suspended. In addition assays of soil microorganisms (total actinomycetes, total bacteria, and actinomycete antagonists of *Fusarium roseum* f. *cerealis* (Cke.) Sny. & Hans.) were made so that the suspension characteristics of both soil and microorganisms could be studied.

(1) Soil Suspension Characteristics

The soil used in these investigations was Wooster silty loam top soil. Moist soil was thoroughly mixed and placed in a 650-ml-capacity screw-lid jar. Soil moisture was determined by oven-drying three 10-g aliquots of the soil by standard procedures. The average percentage moisture of these aliquots was then used to adjust the weights of soil taken for dilution to an oven-dry basis.

Duplicate series of soil weights of 2, 3, 4, 5, 6, 7, and 8 g respectively were each suspended in 100-ml portions of sterile distilled water contained in 1000-ml Erlenmeyer flasks. These weights of soil added to make the dilutions will henceforth be referred to as added soil weight (Asw). The soil suspensions were

[1]Contribution from the Ohio Agricultural Experiment Station (Journal Article No. 61-60), Wooster, Ohio.

shaken 5 minutes on a Burrell Wrist-Action Shaker. From each of these flasks three 5-ml aliquots were withdrawn with a 10-ml serological pipette and distributed in three tared aluminum weighing dishes. The flasks were kept agitated by hand while withdrawing aliquots. The contents of the weighing dishes were then evaporated to dryness on a large electric hot plate, and weighed on a single-pan analytical balance. For each suspension the dry weights of the 5-ml aliquots were averaged, converted to a per ml basis, and recorded as suspended soil weight (Ssw). This Ssw was compared with the appropriate Asw for each series of suspensions. Data from two trials of the duplicate soil suspension series plus a third series, made in triplicate with other Asw values, were combined and an equation was obtained which expressed the relationship of Ssw to Asw. A plot of the relationship is shown in Fig. 1.

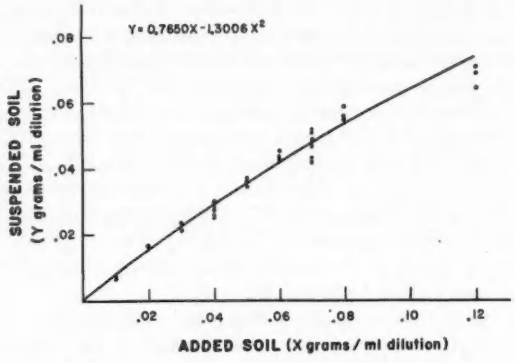

FIG. 1. Relation between weight of soil added and that in suspension using Wooster silty loam. Points represent actual values obtained.

It is evident from the equation that measures of the soil weight in suspension were less than the weights of soil taken to make the dilutions, indicating imperfect suspension of the soil. The equation given above relates Ssw to Asw for the soil type used (Wooster silty loam) and may be applicable only to this specific soil type.

(2) Microbial Dilution Characteristics

The duplicate soil suspension series discussed in the preceding section were diluted for microbial assays in two ways: (1) on the basis of the Asw, and (2) on the basis of the Ssw. Statistical comparisons of the counts obtained by these two differently based dilutions were then made.

Total actinomycetes and total bacteria were assayed using a soil extract isolation medium, made according to Lochhead's formula (2), employing a 1:250,000 dilution. Counts were made following a 2-week incubation of the plates at 25° C. Actinomycetes antagonistic to *F. roseum* were assayed by a triple-agar-layer plate technique (1) employing a 1:50,000 dilution per plate.

The dilution characteristics of soil may not necessarily be the same as the dilution characteristics of microorganisms. It has already been established that the soil determination from suspended material underestimates the actual amount of soil in the preparation. Therefore, if the dilution were made on an underestimated weight, the preparation would be underdiluted and the number of microorganisms would be overestimated. The actual counts from 28 sets of 5 plates each for total actinomycetes and bacteria, and from 28 sets of 10 plates each for antagonists were averaged and are presented in Table I.

TABLE I

Comparison of microbial counts based on added soil weights and on suspended soil weights, with and without correction for suspension error

	Colony counts		
	Added soil (control)	Suspended soil (adjusted)	Suspended soil (unadjusted)
Actinomycetes[a]	95.6	88.1 n.s.	125.7***
Bacteria[a]	240.3	246.9 n.s.	352.7***
Antagonists[b]	67.3	63.1 n.s.	90.1***

[a]Average number per set of 5 plates from 28 sets of 5 plates each.
[b]Average number per set of 10 plates from 28 sets of 10 plates each.
n. s. Difference not significant from control (at 5% level).
***Difference significant from control (at 0.1% level).

It is apparent that the counts from dilutions based on suspended weights overestimated the number of organisms for each of the three categories of microorganisms (see columns 1 and 3).

In employing the dilution procedure proposed in this paper the suspended soil weights would be determined, converted to an "actual" or added weight by use of the equation, and the dilution made on the basis of the calculated "actual" weights (rhizosphere soil sample weights). If this had been done, the counts made from the suspended weights would have been reduced in accordance with the equation relating Ssw to Asw.

To test this procedure the counts based on suspended weights were reduced by factors obtained from the Ssw–Asw weight relationship. An "adjusted" count was thus obtained which represented what presumably would have been obtained had the proposed dilution procedure been used. A comparison of the counts from added weights and "adjusted" counts from suspended weights is given in columns 1 and 2 of Table I.

Obviously the adjustments based on the Ssw–Asw equation were sufficient to bring the counts within the sampling error of the counts based on added weights. It can be concluded that the soil dilution characteristics and microbial dilution characteristics do not differ within the limits of precision inherent in the assays.

The dilution procedure proposed should be applicable to other soil types once the Ssw–Asw weight relationships of the soil types are determined.

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DEPARTMENT OF BOTANY AND PLANT PATHOLOGY,
OHIO AGRICULTURAL EXPERIMENT STATION,
WOOSTER, OHIO.

APPARENT EFFECT ON THE MYCETOMAL MICROORGANISMS OF REPEATED EXPOSURE OF THE HOST INSECT, SITOPHILUS GRANARIUS (L.) (COLEOPTERA), TO METHYL BROMIDE FUMIGATION

A. J. MUSGRAVE,[1] H. A. U. MONRO,[2] AND E. UPITIS[2]

Two laboratory strains (MW and GG) of the granary weevil, *Sitophilus granarius* (L.), kept in Canada, have been separated on microbiological criteria (4). Typical normal weevils of the GG strain harbor great numbers of highly characteristic microorganisms of uncertain identity in the mycetomes of the larvae. The adult weevils, which are dark brown in color when mature, lack mycetomes, and the mycetomal microorganisms are found in the midgut caeca* and in the female gonads (4). However, in the pale brown adults of the MW strain, the mycetomal microorganisms characteristic of the GG are rare or absent though other microorganisms are sometimes evident, as might be anticipated, for both strains of weevils inhabit a heavily contaminated environment and are known to harbor at least three species of bacteria (1).

During the course of other investigations (2) it was noticed that after several generations of selection pressure by methyl bromide, weevils of the GG strain had not only become tolerant to methyl bromide but were light brown in color like typical members of the MW strain.

A comparison with respect to mycetomal microorganisms of GG weevils tolerant to methyl bromide with the control weevils, which were dark brown in color and susceptible to methyl bromide, therefore seemed worth while.

The appropriate organs of weevils were examined as freshly crushed smears from living material using the phase-contrast microscope at ×240 (3), under which conditions the mycetomal microorganisms characteristic of the GG strain were readily seen.

Because the weevils were known to harbor various microorganisms, the presence of which might obscure the state of affairs with regard to the myceto-

[1]Ontario Agricultural College, Guelph. National Research Council grantee.
[2]Fumigation Section, Pesticide Research Institute, Canada Agriculture, London, Ontario.
*Recent observations by A. J. Musgrave indicate that mycetomal microorganisms are present in the mesentera of old weevils (up to 85 days) contrary to a statement of Schneider (5).

mal microorganisms, it was decided to attempt to simplify the recording of results, so that each weevil examined could be classified as either positive or negative. To this end any preparation in which, after a conscientious search of several fields, fewer than five structures that could be regarded as mycetomal microorganisms typical of the GG strain were seen, was classed as negative regardless of other microorganisms that were present. In practice, the distinction was quite clear. Typical GG weevils harbored abundant, easily seen elongate rods.

Results given here were recorded from adult weevils of various ages, including very young specimens, from several generations. "Tolerant" weevils were those whose ancestors had, in several generations, survived at least 11 exposures to methyl bromide. These weevils remained light brown in color for several generations after removal from methyl bromide selection pressure. "Regular" weevils were those whose ancestors had acted as "controls" in the methyl bromide selection experiments. The two strains of weevils were kept under the same conditions which, however, varied from 70% to 76% R.H. and 24° to 28° C. Observations were made on the mesentera only of male specimens, as mycetomal microorganisms are not known from male gonads, and on the mesentera and gonads of female specimens.

Of the 15 males and 23 females of the "regular" strain examined, all 38 were positive; of the 28 males and 32 females of the "tolerant" strain examined, all 60 were negative.

It seems possible to conclude that selected tolerance to methyl bromide is associated with the absence of or transformation of the mycetomal microorganisms.

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**Sitophilus granarius* is used here for the granary weevil in accordance with common N. American usage instead of *Calandra granaria* as employed elsewhere. A complex situation is analyzed by Vaurie (6).

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Ontario Agricultural College,
Guelph, Ontario,
and
Fumigation Section,
Pesticide Research Institute,
Canada Agriculture,
London, Ontario.

A STUDY OF THE LIPID CONTENT OF TRICHOPHYTON MENTAGROPHYTES*

R. C. S. AUDETTE, R. M. BAXTER, AND G. C. WALKER

Studies of the metabolic products of the dermatophytes have only recently been undertaken and the lipids elaborated by several of these fungi have been partially investigated (5, 6, 8, 9).

A 3-day-old conidial suspension of *T. mentagrophytes* 185, grown on Sabourauds' agar, was agitated continuously in a medium consisting of 4% peptone and 8% glucose for 14 days. The resultant mycelial pellets were extracted by petroleum ether (boiling range 38–52) for 20 hours, and after removal of the petroleum ether an orange-brown residue (8 g from 65 g dry mycelium) remained which was refluxed in 10% methanolic potassium hydroxide for 3 hours.

The unsaponifiable fraction yielded a white crystalline material (100 mg from 65 g of dry mycelium) which gave positive Salkowski and Liebermann-Burchard color reactions, indicative of a sterol possessing a diene system. A comparison of the melting points and absorption spectra data of the isolated material, authentic ergosterol, and of the benzoate derivatives is shown in Table I and indicates that the isolated crystalline material is ergosterol.

TABLE I

Melting point and absorption spectra data of the sterol from *T. mentagrophytes*

	Melting point*†		Absorption maxima (mu)	
	Observed	Reported	Observed	Reported
Sample				
Sterol	152–153°		261, 271, 281, 292	
Ergosterol	154–155°	163 (2)	261, 271, 281, 292	260, 271, 281.5, 293.5 (4)
Sterol benzoate	165–168°		226, 261, 271, 281, 292	
Ergosterol benzoate	165–167°	169–171° (4)	226, 261, 271, 281, 292	225, 260, 271, 280, 293 (1)

*Uncorrected, using heated block technique.
†Mixed melting points of the sterol samples and their derivatives showed no depression.

The saponifiable fraction was extracted from the acidified reaction mixture using ether. A brown solid residue (1.15 g) remained after evaporation of the ether *in vacuo*. Methyl esters of the component fatty acids were prepared by refluxing the solid residue for 3 hours with 1% sulphuric acid in methanol.

The methyl esters were analyzed by gas–liquid chromatography using a 10-ft column of diethylene glycol succinate polyester (DEGS). The unit was operated at 202° with a gas flow of 50 ml helium per minute (Wilkens Instru-

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ment and Research). The chain length and degree of unsaturation of the components were deduced from the emergence times for palmitic, stearic, oleic, and linoleic acid. The presence of the other fatty acids was ascertained from a log plot of their emergence times against the number of carbon atoms in the molecule, as described by James (3).

TABLE II

The component fatty acids of *T. mentagrophytes*

Fatty acid	Percentage	Fatty acid	Percentage
Octanoic	0.1	Monounsaturated C-16	0.2
Dodecanoic	0.1	Heptadecanoic	1.0
Tetradecanoic	0.3	Stearic	11.2
Pentadecanoic	0.9	Oleic	17.0
Palmitic	23.8	Linoleic	45.2

The fatty acids present in the lipid fraction of the mycelium of *T. mentagrophytes* are shown in Table II. The percentages of the fatty acids reported are percentages of the total fatty acids present, not of the whole lipid fraction.

The presence of 97.2% of the sample as C-16 and C-18 fatty acids confirms the observation that the fatty acids of *T. mentagrophytes* were predominantly long chain types (7).

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Faculty of Pharmacy,
University of Toronto,
Toronto, Ontario.

MAGNESIUM AS A FACTOR IN DETERMINING THE COMPARATIVE SENSITIVITY OF MARINE BACTERIA TO FOUR TETRACYCLINE ANTIBIOTICS

B. A. SOUTHCOTT AND H. L. A. TARR

During a study of the keeping quality of Pacific coast dogfish (*Squalus suckleyi*) (1, 2) typical bacterial colonies were isolated from a number of plates, and cultures of these isolates were studied by the usual procedures in order to permit a rough classification. When the sensitivity of each culture to four of the tetracycline antibiotics was determined, using sensitivity disks (Bacto, 10.0 μg), it was found that they were less sensitive to the tetracyclines on sea water agar plates containing peptone, yeast extract, $FePO_4.2H_2O$, and artificial sea water (1) than on nutrient agar plates (Difco). It was also observed that the sensitivity to the tetracyclines differed considerably on the two agars and that sensitivity to OTC (oxytetracycline) was invariably lower than that to CTC (chlortetracycline), TC (tetracycline), or DCTC (demethylchlortetracycline). Typical results with five different cultures are given in Table I.

TABLE I

Effect of agar media on inhibitory effect of tetracyclines

Culture	Diameter of inhibitory zone (mm)							
	Nutrient agar				Sea water agar			
	CTC	OTC	TC	DCTC	CTC	OTC	TC	DCTC
1 Gram+ coccus	29	25	29	29	23	9	18	21
5 Gram+ coccus	28	21	25	32	0	0	0	0
51 Gram− rod	12	0	11	11	0	0	0	0
79 Gram+ rod	44	35	41	40	23	0	18	19
109 Gram+ coccus	29	24	26	27	16	0	12	12

In an attempt to identify the substance or substances responsible for the apparent resistance of these cultures to the tetracyclines in sea water agar, a synthetic agar[1] was prepared to which various inorganic salts were added. Tests with typical organisms revealed that, with a single exception, under the experimental conditions, Mg^{++} but not Na^{+} or Fe^{+++} contributed to the increase in resistance to tetracyclines using the synthetic agar. The results obtained using an orange-pigmented Gram-positive coccus isolate (culture 109, Table I) are given in Table II.

Culture 5 (Table I) became completely unaffected by the tetracyclines where sufficient Mg^{++} was added to the medium, the amount of Mg^{++} required to exert this depending on the tetracycline present (Table III).

[1]Composition of synthetic agar per 100 ml: 0.3 g glucose; .09 g $(NH_4)_2SO_4$; .013 g $(NH_4)_2HPO_4$; 1.5 g agar; 100.0 μg each of riboflavin, Ca pantothenate, thiamine HCl, and niacin; 20.0 μg each of pyridoxal hydrochloride and para-aminobenzoic acid; 1.0 μg each of biotin, folic acid, and vitamin B_{12}; 40.0 mg alanine; 20.0 mg glutamic acid; 16.0 mg aspartic acid; 8.0 mg each of arginine hydrochloride, lysine hydrochloride, isoleucine, methionine, phenylalanine, threonine, valine, tryptophane, and serine; 4.0 mg each of histidine, leucine, proline, tyrosine, cystine, and glycine.

These results would seem to afford a possible explanation for the fact that CTC has proved superior to both OTC and TC for fish preservation (3). Fish muscle contains 0.01 to 0.03 *M* Mg^{++} (4). So far, the possible inhibitory effect of Ca^{++}, which occurs in somewhat higher concentrations in fish muscle, has not been explored. The chelating effect of the tetracyclines for divalent ions has frequently been described (5, 6), but results similar to the above do not appear to have been reported. Shellfish such as crab, oysters, and shrimp require higher concentrations of tetracycline antibiotics for successful preservation than do fish (3), and a possible explanation of this could be that shellfish normally have considerably higher concentrations of both Mg^{++} and Ca^{++} ions.

TABLE II

Influence of added salts on inhibitory effect of tetracyclines

Salts added (molar concentration)			Diameter of inhibitory zone (mm)			
			CTC	OTC	TC	DCTC
NaCl	$MgCl_2$	$FePO_4$				
			54	23	40	40
0.1			54	23	40	40
0.2			50	24	40	40
	0.05		36	14	23	32
	0.1		32	14	18	26
		0.00006	50	22	43	43
		0.00012	49	23	40	45
0.2	0.1		36	17	17	29
0.2		0.00012	44	22	42	44
	0.1	0.00012	33	13	19	25
0.2	0.1	0.00012	30	16	18	25

TABLE III

Effect of Mg^{++} concentration on inhibitory effect of tetracyclines

$MgCl_2$ (molar concentration)	Diameter of inhibitory zone (mm)			
	CTC	OTC	TC	DCTC
0	29	11	26	29
0.005	22	9	16	27
0.01	20	9	16	25
0.03	19	Trace	13	21
0.05	17	0	9	21
0.08	17	0	Trace	18
0.1	13	0	0	17
0.3	0	0	0	12
0.5	0	0	0	0

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USE OF A TETRAZOLIUM SALT FOR AN EASILY DISCERNIBLE KCN REACTION

M. GERSHMAN

Møller's KCN medium is a notable contribution to the differentiation of several groups of enteric bacteria (1, 3). KCN sensitivity is particularly useful in the differential diagnosis of *Salmonella* (KCN-negative) from the frequently occurring slow lactose, sucrose, and salicin fermenting strains of *Escherichia freundii* (KCN-positive).

KCN broth base medium is available commercially facilitating its routine use as a diagnostic tool. Tubes are inoculated with one loopful of a 24-hour broth culture, incubated at 37° C, and observed daily for growth for 4 days. Growth, on occasion, may be so slight, however, as to necessitate comparison with a control tube for proper interpretation.

Colorless solutions of various tetrazolium salts, in the presence of viable cells, are reduced to insoluble pigments. Kelly and Fulton (2) made use of this property in correcting the difficulty in discerning growth in semisolids by incorporating tetrazolium salts into the test medium and in this way inducing a cellular formation of red pigments.

Positive KCN reactions, therefore, when they do occur, can be rendered easily discernible by the use of a sterile KCN broth base containing a 0.005%, Seitz-filtered, aqueous solution of 2,3,5-triphenyltetrazolium chloride. Growth is revealed by the presence of a red precipitate.

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DEPARTMENT OF ANIMAL PATHOLOGY,
UNIVERSITY OF MAINE,
ORONO, MAINE.

NOTES TO CONTRIBUTORS

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